STUDIES ON A BIOASSAY FOR PROGESTERONE

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THE UNIVERSITY OF ALBERTA

STUDIES ON A BIOASSAY FOR PROGESTERONE

A DISSERTATION

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

FACULTY OF ARTS AND SCIENCE

by

CHARLES HEATH

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ABSTRACT

Physico-chemical and biological procedures for the assay of progestogens have been reviewed. A procedure based on the characteristic proliferation of the oestrone primed rabbit endometrium in response to intra-uterine implants of progesterone mixed with cholesterol, was investigated and found satisfactory for the estimation of progestogens in amounts as low as one microgram.

Various procedures were used to prepare extracts from blood samples. A number of these extracts, when tested for activity in the rabbit uterus produced proliferation. Using a chromatographic extraction procedure, quantitative recovery of added progesterone was obtained.

The threshold doses of oestrone and of ethinyl oestradiol required to produce oestrogenic effects in the rabbit uterus have been determined for systemic and intra uterine administration of these oestrogens.

The doses of oestrone required to inhibit the uterine progestational response to progesterone, for various conditions of administration of these two hormones were determined.

Various types of implants containing progesterone were tested and none was found more satisfactory than implants of progesterone in cholesterol.

in causing endometrial proliferation. Those made from 0.2% progesterone in cholesterol produced the greatest response with a given dose of progesterone.

Color reactions of progesterone with salicylaldehyde and benzoyl chloride were examined. In preliminary tests the benzoyl chloride reaction appeared satisfactory for estimation of pure progesterone in amounts greater than ten micrograms.

ACKNOWLEDGEMENT

I wish to express my gratitude to the National Research Council of Canada for providing the funds which made this work possible. I am indebted to Doctor E.O. Höhn who directed this work and to Professor H.V. Rice, the head of the Department of Physiology and Pharmacology, for their assistance and many helpful suggestions; to Mrs. H. Mackie who rendered valuable technical assistance; to Mrs. L. Morris and my wife who deciphered my manuscript and typed it. I should also like to thank the other members of the Department of Physiology and Pharmacology for their cooperation and interest in this work.

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NATURE OF THE PROBLEM

Although several methods have been proposed for the estimation of progesterone, for various reasons none have been generally accepted. In this thesis work on a bioassay, based on the response of the rabbit endometrium to the local application of solid progesterone, is reported.

Since this response is modified by the concentration of oestrogen that is present, certain aspects of the interaction of progesterone and oestrogen are also considered.

Finally, as an aid to following progesterone through the stages of its extraction from blood, certain color reactions of progesterone were also investigated, and these are reviewed.

ADDENDA

The following additions and explanatory notes were recommended by the board of examiners.

p. 35, #2. This distillation was carried out at reduced pressure (10 to 15 cm. of mercury).

p. 38, Table 7. The solutions used in the experiments outlined in Table 7 consisted of pure crystalline progesterone dissolved in benzene. Suitable aliquots were then taken and washed with alkali.

p. 43, para. 4. The purpose of these solutions of progressively stronger chloroform concentration was to elute substances less strongly adsorbed on the alumina of the column than was the progestogen.

p. 45, para. 5. It was concluded from the experiments listed in this table that the recovery of progestogen, using the chromatographic procedure, did not appear to be significantly less than that obtained using the modified Corner-Allen procedure previously described.

p. 72, para. 2. It was realized, however, that of the color reactions investigated by Elvidge, none was specific for progesterone, or even for 4-3 ketosteroids (6) (8) (14) (33).

p. 73, Table 26 et seq. In all the following experiments which involve measurement of color, the instrument used was set to zero using a blank which consisted of reagents only.

SECTION I.

INTRODUCTION

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INTRODUCTION

SCOPE AND POSSIBLE APPLICATIONS OF A DETERMINATION FOR PROGESTOGENS

Our present knowledge of the physiological properties of the hormone progesterone has been attained without the assistance of a satisfactory means for its determination in the human bloodstream. It has been shown, for example, (7) (43) that when progesterone is administered, pregnanediol can be recovered from the urine. Also, pregnanediol has been demonstrated in the urine during the luteal phase of the menstrual cycle (42) (45).

In cases where the concentration of substances having progestational activity is abnormal, levels of progestogens can only be inferred by the determination of pregnanediol, a metabolic product to which progestogens are converted; or by examination of the endometrium.

A satisfactory bioassay would permit normal levels to be established directly, during the menstrual cycle and pregnancy. Further, abnormalities such as Metropathia Haemorrhagica in which progesterone is believed to be lacking, could be more specifically diagnosed and more

scientifically treated. Cases of habitual abortion and of sterility due to failure of implantation of the fertilized ovum could be similarly investigated.

A progestational substance (probably progesterone) has been demonstrated in the blood of pregnant women (20) (5). However, its metabolism is not well known. There is evidence that the liver is involved (28) and the kidney (23) (25).

A satisfactory means of determination would enable the fate of the hormone in the body to be established. A paper by Hooker and Forbes which appeared in August, 1950, (23) does, in fact, make use of their mouse assay to investigate the renal inactivation of progesterone.

REVIEW OF METHODS USED FOR THE BIOASSAY OF PROGESTOGENS[1]

A. The first systematic test for progestogens was described by Corner and Allen (9). In this, an adult doe rabbit is mated. If, on examination 18 hours later, ruptured ovarian follicles are found, a piece of uterus is removed for control, and the animal is ovariectomized.

Five daily subcutaneous injections of the extract to be tested are then given and the animal is killed on the fifth day, 24 hours after the last injection. Parts of the uterine cornua are removed, fixed and examined by microscope.

The test is considered positive if the endometrium has attained a degree of progestational proliferation equivalent to that normally attained on the eighth day of pregnancy. The authors define 1 rabbit unit of extract as the amount required to produce this proliferation.

The authors state that presumably mature does will react whether previously mated or not. However, they recommend the above procedure of mating followed by laparotomy to ensure

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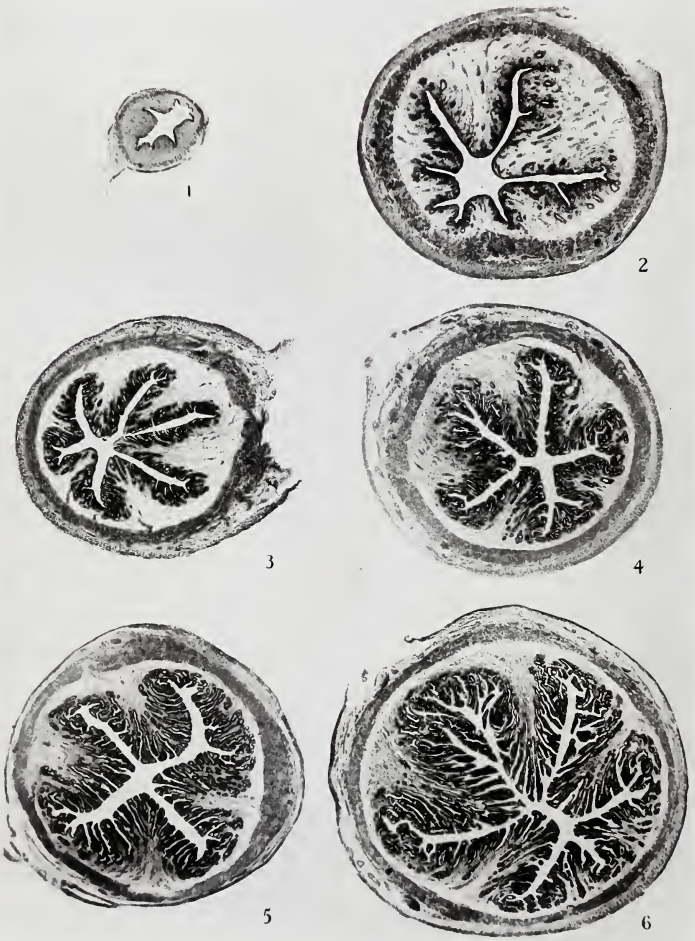
[1]The term "Progestogen" as suggested by Robson in his book "Recent Advances in Sex and Reproductive Physiology" will be used throughout this thesis to refer to substances having a progesterone-like activity. This term is employed in the same sense as the words "Oestrogen" and "Androgen". The word "Progesterone" refers to the specific chemical substance to which are generally ascribed the properties of the hormone of the Corpus Luteum.

FIGURE 1.

THE McPHAIL SCALE OF PROGESTATIONAL PROLIFERATION SHOWING THE EFFECT OF OESTROGEN ALONE, AND OF OESTROGEN AND PROGESTERONE



Uteri of immature rabbits, showing the standard scale of progestational proliferation (×17). Fig. 1, no treatment; fig. 2, œstrin only, reaction 0; figs. 3–6, œstrin followed by progestin, reactions 1, 2, 3 and 4 respectively.

To face p 148

exactly comparable conditions with regard to the reproductive systems of the test animals. They also state that experiments using immature rabbits led to very variable results.

This method for the assay of progestogens is still widely used. However, it suffers from the disadvantage that at least 750 micrograms of progesterone or its equivalent are required to produce a positive response.

B. McPhail (30) stated in 1935 that only two definite tests existed for the hormone of the corpus luteum. These were, "the causation of progestational proliferation of the rabbit uterus and the sensitization of the rat, mouse or guinea-pig uterus to the deciduomata reaction." He then discussed a method for the determination of progestin[1] based on the former, using immature female rabbits.

These rabbits were first injected intramuscularly with a priming dose of 15 micrograms of oestrone dissolved in oil, three doses being spread over six days. Progestin in oil was then injected daily for five days and the resulting endometrial proliferation compared with a series of standards which he had prepared.

This method showed such individual variation between test animals that the author felt it necessary to use groups of at least five animals. The sensitivity was of the order of 0.5 I.U. of progestin (1 I.U. = 1 mg. progesterone).

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[1]"Progestin" is a more or less purified extract containing mainly progesterone.

This investigation, while of little practical use as a method of assay, because of its low sensitivity and variability, did provide the following useful information.

1. An optimal priming dose of oestrone exists which, under the experimental conditions, ranged from 15 micrograms to 150 micrograms.

2. Oestrone administration over a period of 6 days resulted in adequate growth of the uterus, while if the treatment was only for 4 days, even large doses were ineffectual.

3. A given dose of progestin caused the greatest reaction when it was administered over periods of 3 or 5 days, longer periods giving a lesser degree of proliferation.

4. A standard scale of progestational proliferation was described and illustrated. This scale has been used in the present work (see figure 1).

C. In 1939 McGinty et al, also using the immature rabbit uterus, investigated the response to direct application to the endometrium of solutions of progesterone in oil. (29)

The animals were first primed by injection of oestrin; "150 I.U. over 6 days". Then the uterus was prepared by ligation of the upper end of a uterine horn and placement of a second loose ligature three to four centimeters below. Next, 0.1 cc. of lanolin or peanut oil containing the hormone was injected into the lumen of the uterus from below the second ligature which was pulled tight as the needle was withdrawn. The injection was thus retained in a restricted segment of uterus between the ligatures. The rabbits were killed 72 hours

later and sections of the cornua were examined and rated according to the McPhail scale.

The results indicated that amounts as small as 0.125 to 0.25 micrograms of progesterone would give a proliferation. Further, the proliferation was confined to the segment of the uterus that was tied off and contained the injected progesterone.

Although the investigation was apparently carried no further by these workers, the possibility of a highly sensitive method of assay was demonstrated.

Haskins has used the method of McGinty and co-workers for the assay of progesterone in the serum of guinea-pigs (19) and of women (20). In the latter instance, he obtained no reactions stronger than would be produced by the intrauterine injection of 0.25 micrograms of progesterone in oil. He also found that peanut oil alone, when injected, occasionally resulted in proliferation.

D. A very sensitive and specific response of the endometrium of the mouse to progesterone is claimed by Hooker and Forbes and was made the basis of a bioassay. This response consists of hypertrophy of the stromal nuclei following intrauterine injection of minute amounts of progesterone in oil. (22)

In their procedure, the uterus was delivered through a mid-ventral incision and two ligatures were placed around it, a tight one cranially and a loose one caudally. These were held in a jig which effectively served to maintain them 5 mm. apart. Injection was made between them by means of a

specially designed micrometer syringe which was found to deliver regularly 0.6 microliters. After injection, the caudal ligature was tied. Forty-eight hours later the animal was killed and the uterus sectioned. The sections were examined under a microscope.

Amounts of progesterone as low as 0.0002 micrograms gave a consistently positive reaction. Moreover, testosterone and desoxycorticosterone acetate did not elicit the response, and the reaction appeared to be independent of the oestrogen concentration present.

Additional experiments established that it was necessary to spay the mice at least 16 days prior to progesterone implantation, and that the optimal progestational effect was evident 48 hours after implantation.

The following disadvantages are listed by the authors:

1. A great deal of time and labour is required.

2. The response is all-or-none, i.e., it has not been possible to demonstrate different degrees of positive responses.

3. It is very difficult to recognize the positive nuclear response of the test with certainty. This was further borne out by preliminary experiments of Dr. Höhn in which neither he nor I were able to identify the injected uteri without error.

Thus, while this method apparently gives excellent results in the hands of skilled and experienced workers, it

is too difficult to assess and too tedious for general application.

E. Szarka (41) published a method of assay of progestogens based on the inhibition of the uterine manifestations of oestrus in rabbits and rats. Uterine oestrus was determined by assessing the degree of distension of the uterus.

The effect of systemic injection of a standard test dose of oestrogen (4.8 micrograms) in the spayed rat was found to be inhibited by 1.5 milligrams of progesterone. By using immature rats the sensitivity of this method was increased so that a minimal dose of 80 micrograms of progesterone could be detected. This degree of sensitivity is not sufficient for the estimation of progesterone in human blood.

F. The response of the ovipositor of a fish, the bitterling, to progesterone added to its aquarium has been made the basis of an entirely different type of bioassay. Duyvené de Wit (13) found that when amounts of progesterone as small as two micrograms, in solution in propylene glycol were added to the aquarium in which a bitterling was kept, the ovipositor lengthened. It was shown in a series of papers (17) (18) (26) that this response is elicited by a wide variety of substances. This response also shows a marked seasonal variation. However, because this method is so simple to perform, it may yet prove to be useful, provided that the progestogen may first be purified. This could perhaps be done by some physico-chemical procedure such as chromatography.

REVIEW OF PHYSICO-CHEMICAL METHODS FOR THE ASSAY OF PROGESTOGENS

G. A polarographic investigation of a large number of steroids was carried out in 1940 by Wolfe, Hershberg and Fieser. These authors demonstrated that many Δ 4-3, keto-steroids would give a characteristic wave. These substances were suitably treated and then polarographed over the potential range from -1 to -2 volts[1], the span of the waves being then measured. (44)

Three hundred micrograms of progesterone gave a span which corresponded to a current of 6.1 microamperes. However, when progesterone was first treated with Girard's Reagent T, 500 micrograms gave a current of 13.8 microamperes. Thus, the fact that suitable chemical treatment can increase the sensitivity is demonstrated.

These authors showed that the individual concentrations of two polarizable steroids which had different half-wave potentials[2] could be determined in the same solution and

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[1] In polarographic determinations, the concentration of the substance being determined is found to be proportional to the height (span) of the wave.

[2] The half-wave potential is a constant for a given substance and is used as a qualitative test for such a substance when it is known that material having a similar half-wave potential is absent.

at the same time provided that interfering substances were absent.

Six Δ 4-3, ketosteroids, having similar physiological properties were investigated. This series included progesterone. It was found that their half-wave potentials all lay between -1.5 and -1.6 volts. This means that any one of these steroids cannot be determined in a solution in which the others may be present. This disadvantage might be overcome by means of a suitable chemical or physical procedure to remove interfering material.

In the test, as described by these authors, 50 to 100 micrograms of progesterone is the smallest amount which can be determined. This sensitivity could be improved by application of suitable microanalytical technique and by forming a suitable chemical derivative of the progestogen prior to examination in the polarograph. Referring to this paper, Reynolds and Ginsburg state (35) "Müller, who has used the polarographic method in other types of work, believes the method could be developed to the point where 0.001 mg. could be detected with an error of 25-50% and 0.005 mg. with an error of 10%."

H. Reynolds and Ginsburg in 1942 described a determination based on the spectrophotometric absorption at 240 mu which is exhibited by α,β - unsaturated ketones (35). Progesterone belongs to this class of compounds. The absorption is measured in a spectrograph and the unknown concentration is read from a calibration curve, prepared using crystalline progesterone.

As a test of this method the authors added progesterone to 18 samples of 10 cc. of rabbit serum. Each sample was then extracted and the progesterone was determined spectrophotometrically. Six of these samples contained added oestrogen as well as the progesterone, and four samples with no added hormone were also determined as blank controls.

Part of their Table 3 is reproduced below, only those concentrations below 20 micrograms/cc. being shown.

Amount of Progesterone Present (mg./cc.)	Amount of Progesterone Recovered (mg./cc.)	Per Cent Error
0.000	0.000	0
0.000	0.000	0
0.002	0.000	-100
0.010	0.012	+20
0.020	0.022	+10
0.020	0.027	+35
Oestriol as well as progesterone was added to the following		
0.020	0.021	+5
0.020	0.024	+20
0.020	0.028	+40

This method also exhibits lack of specificity in that any Δ 4-3, ketosteroid (which means the presence of the α,β - unsaturated configuration) will exhibit absorption in this region. This class of compound includes testosterone, desoxycortocosterone and others as well as progesterone.

I. A paper chromatographic method was reported in 1950 by Haskins, Sherman and Allen (21). Progesterone dissolved in sesame and cottonseed oils was chromatographed on paper strips for 16 hours, using 80% ethanol as the solvent. The paper was then dried, cut into two strips and the progesterone located on the narrower of these by means of the blue color which resulted on treatment with m-dinitrobenzene and aqueous KOH.

The hormone was then dissolved in ethanol from the untreated strip and determined by ultra-violet analysis in a Beckman Spectrophotometer.

The authors stated that testosterone and desoxycorticosterone acetate gave the same color on the paper but did not give information concerning the rate of movement of these substances and did not deal with the individual determinations of these substances in the same solution. Only if the rate of movement were different could these two steroids presumably be separated by this method.

Less than 5 micrograms of the pure hormones were determined by this method.

J. W. F. Elvidge investigated several color reactions of progesterone and published a method for the estimation of this hormone in solution in ethyl oleate (14). Three of the reactions which he investigated are briefly discussed here.

(1) The Zimmermann Reaction, with m-dinitrobenzene in alkaline solution. The author used the technique of Callow (8) and found the color weak and inconsistent. He did not, apparently, investigate concentrations of progesterone less than 250 micrograms per cc.

This reaction is a general one, commonly used to determine 17-ketosteroids in urine and Elvidge remarks that the extinction values at 520 mu of the color produced by progesterone were less than half that of androsterone (a 17-ketosteroid).

(2) Benzoyl Chloride Method (6). Progesterone gives a green color with this reagent. This reaction was investigated in the course of the work reported in this thesis and is discussed below (page 70).

(3) Salicylic Aldehyde Color Reaction (33). Elvidge finds that this procedure is satisfactory for the estimation of pure progesterone dissolved in ethyl oleate. He does not report experiments where concentrations of less than 50 micrograms per cc. were used nor does he consider in this paper the specificity of the reaction in so far as other steroids are concerned.

In general, it appears that in order to adapt any of these reactions to the estimation of progesterone in biological materials, further investigations will have to be made.

Table 1.

COMPARISON OF SOME METHODS FOR THE DETERMINATION OF PROGESTOGENS

Method	Sensitivity	Specificity	Time Required
A. Corner-Allen	750 ug	Progestogens, precursors	7 days
B. McPhail	500 ug	Progestogens, precursors	7 days
C. McGinty	0.125 ug	Progestogens	6 days
D. Hooker-Forbes	0.0002 ug	Progesterone	4 days
E. Szarka	80 ug	Progestogens, precursors	2 days
F. Bitterling	2 ug	Not specific	< 1 day
G. Polarographic	50 ug	Δ 4-3 keto-steroids	< 1 day
H. Spectrographic	< 10 ug	α,β -unsaturated ketones	< 1 day
I. Chromatographic	< 5 ug	Not stated	2 days
J. Colorimetric	< 50 ug	Probably not specific	--

EVALUATION OF PROGESTOGEN ASSAY PROCEDURES

The procedures reviewed above are listed in Table 1 and several of the advantages and disadvantages of each are compared. In the column headed "Time Required" is an estimate by the writer of this thesis of the time required to extract a tissue sample and to determine its content of progestogen. Bioassays, for example, include time for whatever histological procedures may be necessary.

In considering this table, the following points may be made:

1. In order to make use of a procedure for the assay of progestogens in blood, it must be possible to detect an absolute amount of progesterone of the order of a few micrograms. This rules out methods A, B, E and J. Method G, in the opinion of the writer, may, on further investigation, be improved both in specificity and sensitivity to the point where it will be practicable for clinical use.

2. Method D of Hooker and Forbes, although it appears to be extremely sensitive and specific for progesterone itself, has not yet been independently confirmed by other workers, although it first appeared in 1947. In preliminary trials in this laboratory, it has proven difficult to assess.

3. Method C of McGinty, while quite sensitive,

gives a variable response. This necessitates the use of several animals in estimating the progestogen content of an unknown sample, and hence requires a larger original sample than would otherwise appear to be necessary. Further trials by independent workers will be necessary in order to assess fully its possibilities.

4. The physico-chemical procedures G, H and I are either sufficiently sensitive as presently described, or may be improved until the required sensitivity is attained. However, in using them to determine substances which have the physiological actions of progesterone, it is necessary to be certain that no substance which produces the same physico-chemical reaction but is not a progestogen is present in the extract being tested. If a procedure existed for the quantitative isolation of progesterone from a blood extract, the above requirement would be met. However, no such procedure could be discovered in the literature.

REVIEW OF PROCEDURES USED FOR THE EXTRACTION OF PROGESTERONE FROM TISSUES

A. Allen (2) outlined a procedure which had been used successfully in his laboratory for the extraction of progesterone from luteal tissue of sows. This procedure which has been used as the model for our present work, included the following steps:

1. The tissue (which had been preserved in 95% alcohol) was extracted in a Bloor Extractor, using the preserving alcohol as the extracting fluid. After three such extractions, the author could not demonstrate progestational activity in the residue.

2. The alcohol was distilled off under reduced pressure until a thick sludge remained. This was extracted three times with ethyl ether.

3. This ether solution was concentrated to about 1/3 its volume and the phospholipids precipitated with four volumes of acetone and filtered off. The precipitate was dissolved and reprecipitated once, after which no activity could be demonstrated in the residue.

4. The combined filtrates were distilled to a thick oil in vacuo.

5. This oil was dissolved in absolute methanol and

water was then added to make the concentration of methanol in the final solution 70%. This solution was chilled at -4° and a precipitate which consisted of cholesterol and some fats was filtered off. This operation was repeated three times.

6. The residue, after the evaporation of the solvent still in the form of a thick oil, was dissolved in ether and washed with dilute $NaHCO_3$ to remove fatty acids. (This procedure would also remove oestrogens.) The washings were extracted with ether after acidification and this ether solution was then washed as before and added to the previous extract.

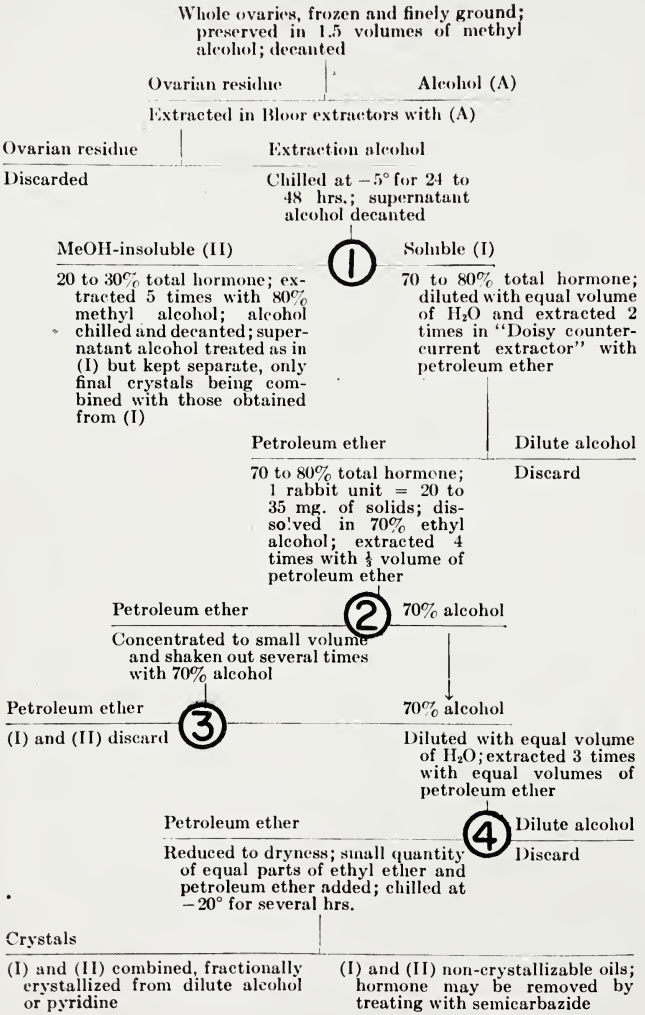
After concentration of the ether solution of stage 6, a thick oily product was recovered from which some crystals could sometimes be obtained with further treatment. Since the recovery of these crystals was variable and incomplete, this further step is not discussed here. The reader is referred to the original paper (2).

In discussing extracts prepared using this procedure, Allen stated, "the purified progestin does not produce heat in rats unless given in fairly large doses ..... even in the crude state one rabbit unit of progestin does not contain over 0.5 to 1 rat unit of estrin." Since oestrogens have been shown to be soluble in dilute aqueous alkali (34), it may be assumed that they would largely be removed by the alkaline wash in stage 6. The importance of considering the fate of oestrogens in a procedure for the extraction of progestogens will be considered in the section of this thesis immediately following and dealing with the antagonism of oestrogens and progestogens.

FIGURE 2.

PROCEDURE OF ALLEN AND GOETSCH FOR THE EXTRACTION OF PROGESTERONE FROM OVARIAN TISSUE

Diagram 1



B. Further improvement of the extraction procedure of Allen has been indicated in a subsequent paper by Allen and Goetsch (4). The authors eliminated several stages which involved distillation and precipitation procedures (stages 2 to 6 of the procedure discussed above). These were replaced by the method outlined in the flow-sheet, Figure 2.

The essential stages of this procedure are as follows:

1. Extraction of the progestogen from the alcohol used to preserve the tissue. (This alcohol was also used to extract any progestogen which remained in the tissue.) This is accomplished by using a continuous counter-current, liquid-liquid extraction apparatus, the design of which appears in their paper (see (1) in Figure 2 opposite).

2. When the partially purified material thus obtained is distributed between petroleum ether and 70% ethanol, additional impurities are removed in the ether and the hormone remains in the alcohol ((2) in Figure 2 opposite).

3. At (4) the alcohol containing the progestogen and some further impurities is diluted. This alters the distribution ratio of the progestogen which may now be extracted by the ether. Thus, a still greater degree of purification may be obtained.

By making use of suitable micro liquid-liquid extraction apparatus such as the one designed by Heberling and illustrated in a paper by G. H. Morrison (32), the above procedure could be adapted to be used in the determination of progestogens in blood.

BEARING OF THE ANTAGONISM OF OESTROGENS AND PROGESTERONE ON THE PROBLEM

The fact that progestogens will cause endometrial proliferation was demonstrated by Corner and Allen (9). These authors noted that in mature doe rabbits which had been spayed, injection of progestin always produced a typical proliferation of the endometrium. However if the rabbits that were used for the test were immature, the proliferation was frequently absent or poorly defined. It is possible that in the immature animals the ovaries had not yet begun to secrete oestrogens, and that this fact is responsible, in part at least for the refractoriness observed in some of the rabbits.

McPhail (31) established that the maximal proliferation was obtained when an injection of a progestogen was preceded by the injection of an optimal amount of an oestrogen. When either more, or less, than this optimal dose was given, little or no effect was apparent from an injection of progestogen. He published photographs illustrating the action of oestrogen alone, and of oestrogen followed by progestin on the uterus of the immature rabbit (see page 4). It will be seen from photo No. 2 that the administration of oestrogen alone does not produce the feathery development of the glands that is caused by progestogens. (photos 3 to 6)

Allen demonstrated (3) that 675 rat units of oestrin would completely inhibit the production of a typical rabbit endometrial response to three mg. of progesterone. However 1000 units did not prevent the formation of normal-appearing corpora lutea, i.e. did not visibly affect the ovarian changes that result in the production of progesterone. Thus oestrin inhibits the actions of progesterone on the uterus but does not interfere with the formation of progestogens in the corpora lutea.

Courrier (10) showed that 0.02 mg. of oestrone would prevent 1.5 mg. of progesterone from producing this typical proliferation of the rabbit endometrium when both were given by injection. The ratio of oestrone to progesterone in this case was 1:75.

In similar experiments on rabbits Robson (37) found that 0.75 mg. of progesterone was completely inhibited by 1/75 its weight of oestrone. He also found that 1/300 its weight of oestrone still gave some inhibition.

Gilman and Stein (16) confirmed the data of Robson and Courrier but showed that the ratio of 1/75 quoted above applied only when the dose of progesterone administered to rabbits was near the smallest dose required to elicit a complete endometrial response[1]. When they injected 8 mg. of progesterone (approximately ten times the optimum[1] dose) a

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[1]optimum dose of progesterone = smallest dose which produces complete endometrial proliferation.

much smaller proportion of oestrone was required to inhibit its action on the endometrium. In this case an oestrone-progesterone ratio of 1:200 gave complete inhibition and 1:1000 partial.

Courrier (11) demonstrated that pregnenolone, another progestogen, could be similarly inhibited by oestrone. He also showed (12) that oestradiol applied locally would inhibit progesterone given systemically. He dissolved the oestrogen in oil and injected it between ligatures into the lumen of the rabbit uterus. Moreover he obtained complete inhibition of the progestational response when he used an oestrogen-progesterone ratio of 1:100. This indicates that when oestrogen is applied directly it may be antagonistic to progestogen in a dosage smaller than when it is systemically administered.

Thus we find:-

(1) An optimal priming dose of oestrogen potentiates the endometrial response to progestogen.

(2) If the priming dose of oestrogen is greater, or less than this optimal dose, this endometrial response to progestogen is reduced.

(3) The endometrial response to an optimum dose of progesterone can be inhibited by 1/75 its weight of oestrone simultaneously administered.

(4) If the dose of progesterone is higher than the optimum a smaller proportion of oestrone than 1/75 is required to inhibit it.

(5) It is indicated that oestrogen applied directly to the endometrium is more effective in antagonizing the proliferative response to progesterone than when it is systemically administered.

When it is desired to assay an unknown sample of blood for the progestational action that it might produce, the presence of oestrogen must be considered. If the oestrogen should be removed during the extraction of the progestogen the progestational effect obtained from the blood sample would be an erroneous representation of the response which might result before the sample was extracted.

Similarly if it were desired to estimate the progestogen content of an unknown sample and oestrogens remained in the implants prepared from the sample, false results might be obtained unless the level of the oestrogens was also taken into account.

Because of the antagonism described above between oestrone and progestogens assays for progestogens using the endometrial response of the rabbit were performed on spayed animals, previously primed with a standard dose of oestrone.

SECTION II.

EXPERIMENTAL WORK

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EXPERIMENTAL WORK

BASIS OF THE RABBIT ENDOMETRIUM ASSAY

In the clinical application of any progestogen assay, it is the estimation of progestational activity of substances contained in a blood sample that is of the greatest importance. Thus it was considered that the primary objective of the present work would be to outline a method whereby such activity in blood could be determined within reasonable limits. A biological procedure which depended upon progestational activity for its end-point appeared likely to offer the best prospect for achievement of this objective.

An additional objective which is also of considerable importance is the possibility of improving a progestogen assay so that it would be specific for progesterone itself. This would extend its usefulness in various researches, some of which are suggested in the introduction to this thesis.

This latter aim is complicated by the fact that a number of substances have properties similar to those of progesterone. This is particularly true when some physical or chemical property of this hormone is made the basis of an assay. When, however, progestogen determination is based

on the progestational response, the number of known substances which can also produce this response is limited to a half-dozen or so compounds. Moreover the substances which interfere in such a manner with the bioassay are all less active in producing endometrial proliferation that is progesterone.

McGinty and his associates (29) applied progesterone directly to the endometrium by injecting it in oil into the lumen of the uterus. He kept this oily material localized by two ligatures which encircled the uterus proximally and distally to the site of injection.

When injected in this way, less than a microgram of progesterone usually gave a proliferation. However this response did prove to be somewhat variable so that the test had to be repeated in several animals in order to assay an unknown sample.

It was hoped that by inserting progesterone in solid form into the lumen of the uterus, the sensitivity of the McGinty procedure would be retained while the variability would at the same time be reduced.

Preliminary experiments performed by Dr. Hohm indicated that while the implantation of pure crystalline progesterone into the rabbit uterus did result in proliferation of the endometrium, a fairly large amount of the hormone was required. This could be explained by the hypothesis that a small amount of pure progesterone implanted into the uterus

is quickly absorbed and metabolized. Thus its presence at the cells of the endometrium is too short-lived to produce a recognizable response. Such a hypothesis is supported by the recent paper of Riegel (36) dealing with the metabolism of Radioprogesterone in mice and rats.

Shimkin and White (40) published a method for rendering the absorption of hormonal implants slower and more uniform. They fused the hormone with cholesterol and implanted the resulting pellets.

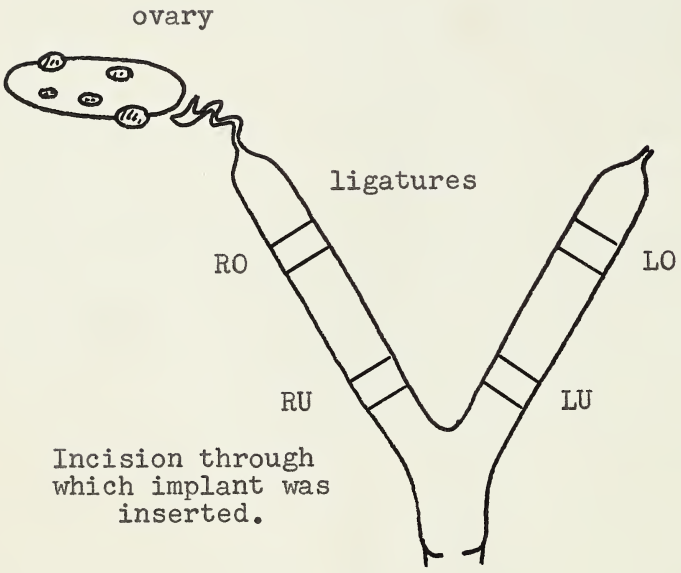
It was decided to follow this procedure and in view of the microgram quantities of progestogen with which it was expected to be dealing a one per-cent mixture of the hormone in cholesterol was used. This mixture was solid and varying doses were obtained by selecting pieces of it containing the appropriate weight of progesterone.

Procedure Mature doe rabbits were spayed. They were then primed with seven d aily subcutaneous injections of ten micrograms of oestrone in olive oil in preparation for implantation.

Under ether anaesthesia the uterus was exposed by a ventral mid-line incision. Ligatures were placed as shown in Fig.3, those nearest the mid-point of each cornu being left loose. Care was taken to insure that at least one artery entered the segment between the ligatures.

FIGURE 3

DIAGRAMMATIC REPRESENTATION OF THE IMPLANTED RABBIT UTERUS



This diagram shows a uterus containing four implants. Those in the right horn are designated RO and RU, those in the left horn LO and LU.

Implants of the test substance were then inserted with forceps so as to lie between one or other pair of ligatures. Those implants which were too small to handle with forceps were placed by first inserting them in a hollow tube fitted with a plunger. The tube was then inserted into the lumen and the implant extruded into the uterus so that the ligature previously left loose could then be drawn tight leaving the implant in an isolated segment 8 - 10 mm long. Four such implants could usually be inserted in the rabbit uterus (Fig.3) and occasionally as many as seven have been placed in the two cornua.

The animals were killed after the implants had remained four days in situ. The implants were then removed, dried and weighed and the uterine segments were fixed in formalin overnight. A control segment was removed from an unimplanted portion of one of the uterine cornua and similarly treated. The next day all these segments were serially passed through three changes of acetone, remaining thirty minutes in each to dehydrate them. The acetone was replaced by benzene which was changed after thirty minutes. This cleared the tissues which were next embedded in paraffin blocks and cut with a microtome into sections 10 microns thick.

The resulting slides were stained with haematoxylin and eosin and examined under the low power of the microscope. The degree of proliferation was then assessed according to the McPhail scale. This scale is illustrated in Fig.1. (facing page 4) by photos 3 to 6 inclusive. These are

Table 2.

RESPONSE TO INTRA UTERINE IMPLANTS OF

1% PROGESTERONE (P) IN CHOLESTEROL

Dose P (ug.)	No. Giving Responses					Total No. Implants	Total No. Positives	Percent Positive Obtained
	0	1	2	3	4			
0.5	8	-	-	-	-	8	0	0
1	8	3	2	-	-	13	5	38.5
2	9	7	3	1	-	20	11	55.0
3	6	5	5	1	-	17	11	64.7
4	3	7	4	2	-	16	13	81.3
5	12	12	11	5	3	43	31	72.2
10	7	6	16	9	13	51	44	82.3
20	1	0	1	4	7	13	12	92.3
25	2	0	3	4	8	17	15	88.3
						199		

rated by McPhail as responses of 1 to 4 respectively. Intermedidate responses are designated by using the figure ½ (e.g. a response greater than that shown in photo 3 but less than that of photo 4 is rated 1½).

The slides of the uterine sections were read independently by Dr. Höhn and the author. They were then graded by each observer independently. The results were compared and it was found that there was invariable agreement within a grade of 1 and usually within a grade of ½, (McPhail scale). Consequently results of experiments previously conducted by Dr. Höhn are included in the statistical treatment to follow.

Some of the sections showed the typical proliferative response only in parts. This was attributed to the relative insolubility of the implants and hence the more localized effect of the hormone. Such sections were rated as 0 - ½ or ½ - 1 etc. depending upon the effect.

Finally, in some of the sections, some effect from the implants was apparent to the experienced eye, although we were not prepared to rate the response as 1. To indicate this fact the value of ½ was assigned to these sections. However in the statistical treatment of the results all ½ responses were considered as zero. All those in which the figure 1 (or a higher number) appeared, e.g. 0 - 1 or ½ - 1, were considered positive.

Table 2 shows the responses to the various dosages used. This table includes the results from all of the pieces

Table 3.

RESPONSE TO INTRA UTERINE IMPLANTS OF

1% PROGESTERONE (P) IN CHOLESTEROL

Data only from those experiments where the residue of the implant was recovered.

Dose P (ug.)	No. Giving Responses					Total No. Implants	Total No. Positives	Percent Positive Obtained	Original and Residual Implant Wt. (mg.)[1]
	0	1	2	3	4				
0.5	1	-	-	-	-	1	0	0	0.05-0.19(1)
1	3	1	1	-	-	5	2	40	0.1-0.9(3)
2	1	5	1	-	-	7	6	83	0.2-0.27(7)
3	3	4	2	1	-	10	7	70	0.3-0.28(6)
4	1	6	2	1	-	10	9	90	0.4-0.36(9)
5	3	8	2	4	3	20	17	85	0.5-0.40(10)
10	1	4	15	5	2	27	26	96	1.0-0.86(22)
20	0	2	2	3	1	8	8	100	2.0-1.73(6)
						87			

[1]The figures in brackets refer to the number of weighable residues on which the weight of the recovered implant is based.

FIGURE 4.

RESPONSES FROM ALL PROGESTERONE-CONTAINING CHOLESTEROL IMPLANTS

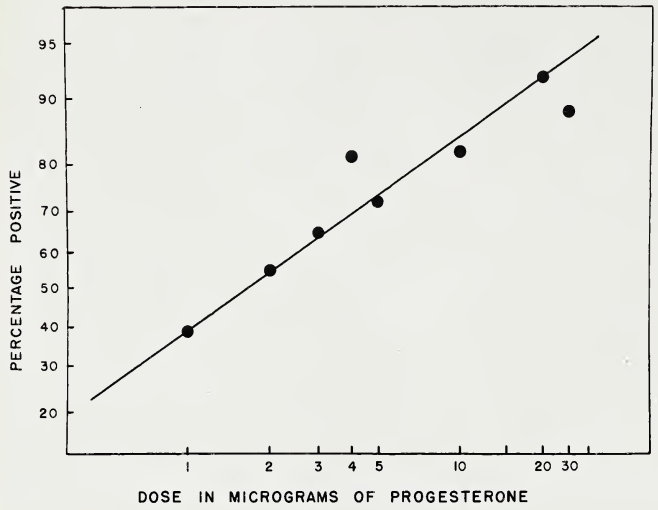
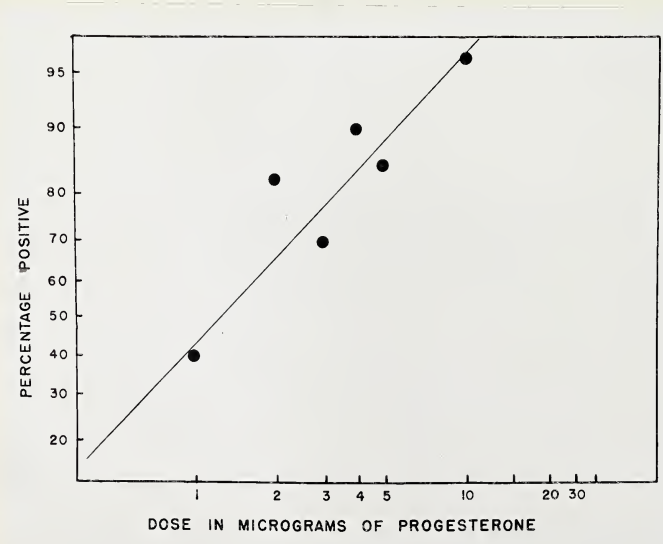


FIGURE 5.

RESPONSES FROM PROGESTERONE-CONTAINING IMPLANTS, EXCLUDING INSTANCES WHERE NO IMPLANT WAS RECOVERED



of 1% progesterone in cholesterol that were implanted. However it was noted that many of the implants of smaller size (mainly those which contained less than 5 micrograms of progesterone) were not recovered when the uterine segments were removed from the animal.

It was realized that loss of some of these implants from their sites of implantation could significantly alter the threshold calculated from this data. Therefore it was decided to compare the threshold calculated from only those experiments in which the implant residues were recovered, with that obtained from all of the data.

Table 3 lists only those experiments in which the implant residues were recovered.

The results expressed in these two tables were submitted to statistical analysis using the method of Litchfield and Wilcoxon (27), for the approximate evaluation of dose-effect experiments. The calculations are presented briefly below but the reader is referred to the original paper for a detailed explanation of each step and for the nomographs used to obtain some of the figures.

The doses of progesterone were plotted against the percentage of positive responses obtained, using Logarithmic-Probability paper. The straight line which appears to be the best fit is drawn in. Figures 4 and 5 show the graphs obtained from the data in Tables 2 and 3. From these graphs the expected percentage of positives is read and the contributions to $(chi)^2$ of each point that was experimentally obtained, are listed. To obtain these values

Table 4.

STATISTICAL DATA FROM FIGURE 4.

Dose P (ug.)	Percent Positive Obtained	Expected Percent Positive	Difference	Contributions to $(chi)^2$
0.5	0	17.0	17.0	0.195
1	38.5	38.5	0	0
2	55.0	54.5	0.5	0
3	64.7	63.5	1.2	0
4	81.3	69.5	11.8	0.065
5	72.2	73.7	1.5	0.001
10	82.3	85.0	2.7	0.005
20	92.3	92.3	0	0
25	88.3	94.0	5.7	0.06
Total Contributions to $(chi)^2$ = 0.141				

Table 5.

STATISTICAL DATA FROM FIGURE 5.

Dose P (ug.)	Percent Positive Obtained	Expected Percent Positive	Difference	Contributions to $(chi)^2$
1	40	43	3	0.004
2	83	65	18	0.120
3	70	78	8	0.004
4	90	83	7	0.003
5	85	88	3	0.007
10	96	97	1	0.003
Total Contributions to $(chi)^2$ = 0.141				

the nomographs from the paper referred to above are used.

Tables 4 and 5 list these figures, which are obtained from the data in Tables 2 and 3 respectively.

Calculations for Data from Table 4

Number of dose levels used = 9

Therefore number of degrees of freedom = 7

Total number of doses implanted = 199

$(chi)^2$ for the line

$$= \frac{0.326 \times 199}{9} = 7.2$$

From the Table of Litchfield & Wilcoxon;

$(chi)^2$ for 7 degrees of freedom = 14.5

Since $(chi)^2$ for the line is less than this value, the line may be considered as a satisfactory representation of the data.

From the graph (Fig.4) the following values were read:[1]

$E.D._{16}$ = 0.28 micrograms

$E.D._{50}$ = 1.65 micrograms

$E.D._{84}$ = 9.4 micrograms

$$S = \frac{1}{2}\left(\frac{E.D._{84}}{E.D._{50}} + \frac{E.D._{50}}{E.E._{16}}\right)$$

$$= \frac{1}{2}\left(\frac{9.4}{1.65} + \frac{1.65}{0.28}\right) = 5.8$$

---

[1] i.e. the dose which gives a positive response in the percentage of implants indicated by the subscript.

N' = Number of doses between $E.D._{16}$ and $E.D._{84}$

= 161

$f(E.D._{50}) = S^{2.77/\sqrt{N'}} = 5.8^{0.219}$

= 1.41

Confidence Limits for $E.D._{50}$

Upper = $E.D._{50} \times f(E.D._{50})$ = 2.33 micrograms

Lower = $E.D._{50} \div f(E.D._{50})$ = 1.17 micrograms

Calculations for the Data from Table 5

Number of dose levels used = 6

Therefore number of degrees of freedom = 4

Total number of doses implanted = 87

$(chi)^2$ for the line

$$= \frac{0.141 \times 87}{6} = 2.08$$

From the Table of Litchfield & Wilcoxon;

$(chi)^2$ for 4 degrees of freedom = 9.49

Since $(chi)^2$ for the line is less than this value, the line may be considered as a satisfactory representation of the data.

From the graph (Fig. 5) the following values were read:

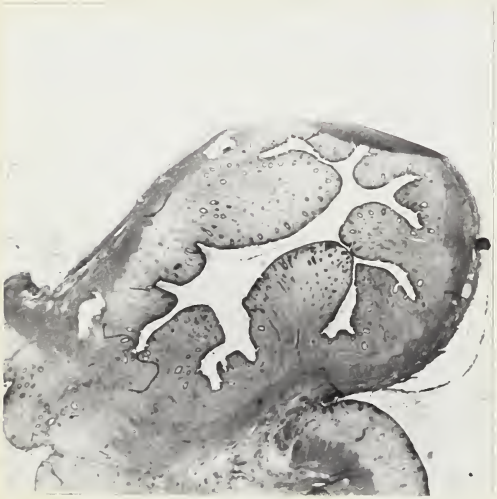
$E.D._{16}$ = 0.28 micrograms

$E.D._{50}$ = 1.25 micrograms

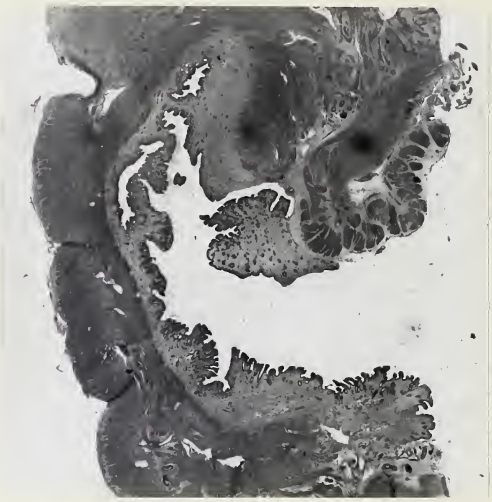
$E.D._{84}$ = 3.95 micrograms

FIGURE 6.

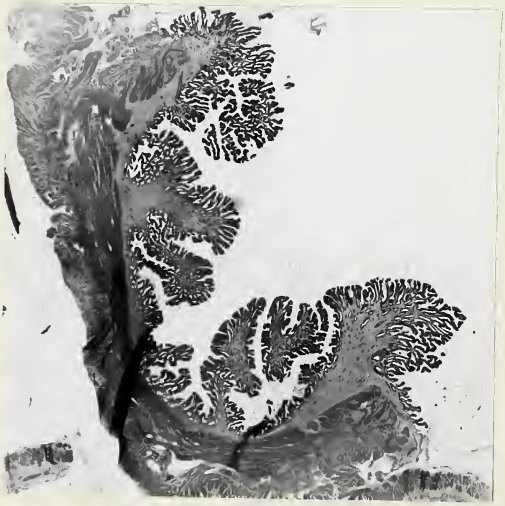
PHOTOMICROGRAPHS SHOWING PROLIFERATIVE RESPONSE TO IMPLANTED PROGESTERONE
(X 25)



A response of 0



A response of 1



A response of 3

$$S = \frac{1}{2}\left(\frac{3.95}{1.25} + \frac{1.25}{3.95}\right) = 3.45$$

$$N' = 33$$

$$f(E.D._{50}) = 3.45^{0.66} = 2.2$$

Confidence Limits for $E.D._{50}$

Upper = 2.75 micrograms

Lower = 0.57 micrograms

Thus we may say, with a 95% probability of being correct, that the dose which will produce a proliferative response in 50% of the animals implanted lies between 1.17 and 2.33 micrograms. These values were obtained by considering all of the progesterone doses implanted in the test animals.

When only the implanted doses from which residues were recovered, were considered, the confidence limits were 0.57 and 2.75 micrograms. The uncertainty is greater because these figures were based on fewer data. The calculated $E.D._{50}$ is 1.25 micrograms in this latter case as compared with 1.65 micrograms for the former.

Typical responses, obtained in the work discussed above are illustrated in Fig. 6. The photomicrographs show:

(1) A response rated as 0

(2) A response rated as 1

(3) A response rated as 3

In assessing these results, it must be realized that the sources of variability are multiple. In addition to the technical errors inherent in the procedure of implantation as described above, there is also varying individual sensitivity to progestogens, including occasional refractoriness. It is not considered however, that variation in the individual response to the priming dose of oestrogen is significant in the final evaluation of uterine sections. This is so because the priming oestrogen causes the uterus of the test animal to become greatly enlarged and hyperaemic. This condition is easily recognized and no animal in which it was not fully developed at the time of implanting would be used to assay an unknown sample.

Errors due to individual variation in sensitivity to progestogens may be minimized by using the procedure suggested below for the assay of unknown samples. Essentially this consists of placing implants of known progestogen content in the uterus of the same animal used to assay the unknown material. It has been noted by the author that variation between various segments in different parts of the same uterus is slight, although no experiments designed to test this point have been performed.

Suggested Procedure for Samples Containing Unknown Quantities of Progestogen

Into one uterine horn are placed two implants of a 1% mixture of progesterone in cholesterol, weighing 0.2 and 1 mg. respectively. These can be weighed accurately and rapidly on a torsion microbalance.

Two suitable implants are prepared from the unknown extract or mixture, their weights being in the approximate ratio of 1:10. These are placed in the other horn. The rabbit is then treated exactly as were those used to obtain the dose-response curve. The resulting sections are then interpreted in the light of the known control implants in the same animal.

The use of two doses of each of the known and unknown mixtures permits a more accurate estimation of the progestogen content, based on the degree of proliferation.

Since this assay is based on the response to a progestogen that is mixed with cholesterol, it is necessary to add cholesterol to an unknown extract in order to reproduce the experimental conditions that apply to the control segments. An arbitrary amount is usually added although the source of the material may furnish a clue to the progestogen level and hence to the cholesterol required. Should the assay indicate results greatly different from this level, a second determination will have to be performed, adjusting the amount of cholesterol added.

EXPERIMENTS DEALING WITH THE EXTRACTION OF PROGESTOGENS FROM TISSUES

Most of the procedures used for the extraction of progestogens that have been reported, are modifications of the one used by Allen (1) in his original work on the hormone of the Corpus Luteum.

It was realized that an extraction procedure which was to precede an assay for progestogens would have requirements differing from those of Allen in the following respects:-

Allen was attempting to isolate a highly purified single chemical substance, or group of substances, in order to establish their identity and further properties. He had available large amounts of tissue with which to work. Moreover he could accept large and variable losses of hormone in order to obtain further purification.

In a bioassay, on the other hand, the size of the sample would be limited. If the assay were to be used clinically, for example, a blood sample would be limited to approximately ten cubic centimeters. The recovery of progestogens from this sample must either be complete, or predictable, so that a factor may be applied to correct for losses. Furthermore the procedure used should not be excessively long or tedious. A complex procedure would be

likely to introduce errors greater than errors due to impurities which remained in the extract.

A. Experiments Using Modifications of the Corner-Allen Procedure for Progestogen Extraction

Preliminary experiments were performed using the following procedure:-

1. The tissue, in a finely divided state was extracted in a Soxhlet apparatus with 95% alcohol.
2. The alcohol was distilled off, stopping the process at the point where only a thick sludge remained.
3. This oily sludge was extracted three times with ethyl ether, the three portions of the solvent being combined and concentrated to one-third their volume.
4. The phospholipids were precipitated with 4 volumes of acetone.
5. This precipitate was dissolved and reprecipitated once more with acetone.
6. The filtrates from stages 4 and 5 were combined and the solvent distilled off, leaving an oily residue.
7. This residue was redissolved in ether and washed with a saturated $NaHCO_3$ solution.
8. The washings were made acid with HCl and extracted with ether.
9. The ether from stage 8 was again washed with saturated $NaHCO_3$ solution and combined with that from stage 7.

10. Cholesterol was added. (99 times the estimated progesterone content of the sample to make a 1% mixture.) The ether was then evaporated off and the waxy residue scraped together to make several implants of varying size.

Where this procedure is modified in preparing the extracts tabulated below this is indicated and details of the modified procedure are listed.

A number of extractions were carried out by Dr. Hohn before the author began work on this problem and the results from these are included in the following tables. Such experiments are marked in the tables by placing the sign (#) before the number of the rabbit in which the extract was implanted. (e.g. # A64).

Table 6.

PROGESTATIONAL RESPONSES TO IMPLANTS MADE FROM TISSUE TO WHICH PROGESTERONE HAD BEEN ADDED

Rabbit No.	Tissue Extracted	Uterine Response	Prog. Content of Implant[1]
#A67	Rabbit muscle	0	100 ug.
#A73	Sheep heart muscle	0	40 ug.
#A65	Rabbit muscle	4 4	20 ug. 20 ug.
#A74	Heart muscle Rabbit muscle	2 2	unknown 10 ug.
A93	Male human blood (alc. extract only)	1 1.5 0.5	2 ug. 4 ug. 10 ug.
175	Rabbit plasma (alc./ether extract only)	0 - ½ 1 1	15 ug. 45 ug. 375 ug.

[1]Calculated from the amount of progesterone added to the tissue.

Extracts Prepared from Tissues to which Progesterone Has Been Added

The results of these experiments are shown in Table 6. The various extracts used will be referred to by the respective numbers of the rabbits in which they were implanted. Extracts A67 and A73 were carried only to stage 3, at which point cholesterol was added and implants made. No explanation can be offered for the complete lack of progestational activity that was exhibited by them other than that of inexperience in extraction procedures.

Extract A74 was boiled with dilute alcoholic alkali, (4 drops of saturated NaOH to 6 cc. of 50% ethanol.) after completing stage 3 of the extraction procedure. The solution was then cooled, shaken with benzene and the implant made by adding cholesterol to the benzene fraction which was then evaporated. Allen suggests (2) that the treatment with alkali would lead to some loss of progesterone and it is perhaps significant that only a proliferation of 2 was obtained from this implant.

Extract A93 was extracted with alcohol only and 175 with a 3:1 mixture of alcohol and ether. Both of these extracts yielded implants that were oily and difficult to handle and in both the results indicate that the distribution of progestational activity was irregular.

Experiments Dealing With the Alkali Treatment of Progesterone Extracts

In order to obtain more information on the effect of alkali treatment of extracts containing progesterone, the experiments listed in Table 7 below were performed.

Table 7

THE EFFECT OF WASHING SOLUTIONS OF PROGESTERONE WITH ALKALI DISSOLVED IN WATER

Rabbit No.	Procedure Followed	Uterine Response	Prog. Content of Implant
# A64	Washed with N/20 NaOH	4	24 ug
	Washed with $NaHCO_3$ (saturated)	3	24 ug
# A74	Washed with N/20 NaOH	4	24 ug

The experiments demonstrate that alkali washing as in the method of Allen (see page 35) does not completely destroy progesterone. Nevertheless even if half of the progesterone originally present were inactivated, it would still have been possible to obtain a response of 4 from the remainder.

Extractions of Ovaries Containing Active-Appearing Corpora Lutea

Two attempts were made to extract progestogens from the ovaries of pregnant rabbits. When uterine implants were

Table 8

THE PROGESTATIONAL EFFECT OF EXTRACTS PREPARED FROM THE BLOOD OF HUMAN MALE SUBJECTS

Rabbit No.	Extract Implanted	Uterine Response	Equivalent Blood Volume
# A66	43 mg. blood extract 10.5 " "	½ 1	43 cc. blood 10 cc. blood
A70	10 mg. blood extract 5 " " "	½ 0	1 cc. blood ½ cc. blood
B61	70 mg. blood extract	0	2 cc. blood
B62	98 mg. blood extract	0	6 cc. blood

Table 9

PROGESTATIONAL EFFECT OBTAINED FROM EXTRACTS OF THE BLOOD OF A PREGNANT HUMAN FEMALE SUBJECT

Rabbit No.	Sample	Uterine Response	Equiv. Blood Volume of Implant
# A33	2 nd week of pregnancy	1 4	1 cc. 3 cc.
# A42	4 th week of pregnancy	0 ½ 0	2 cc. 1 cc. ½ cc.
# A64	2 nd month of pregnancy	1.5 2 1.5	2 cc. 1 cc. ½ cc.
# A66	more of extract A42	1	1 cc.
B27	14 th week of pregnancy	1.5 1	1.cc. ½ cc.
B30	10 th week of pregnancy	½ - 1	1 cc.
B33	14 th week of pregnancy	½	½ cc.
B36	16 th week of pregnancy	0	unknown[1]

[1]Part of this implant, which was oily, was lost while it was being implanted.

made from these extracts, no proliferation could be detected. This result would be obtained if the oestrogens which would also be extracted from the ovaries were in a concentration sufficient to inhibit the progestogens present.

Extractions of Blood Samples from Human Male Subjects

Several samples of blood from human male subjects, which it was considered were not likely to contain progesterone were extracted and tested in the rabbit uterus for progestational activity. The results are indicated in Table 8.

It will be seen from this table that large volumes of male blood appear to contain a small amount of progestogen.

Extractions of Blood from a Human Female Subject (B.C.H.)

All the extracts listed in Table 9 were prepared using the procedure on page 35.

The proliferative response to all these extracts, with the exception of A33 and A64 was weak. This could result from the following circumstances:-

(i) Progestogen is lost or destroyed in the extraction procedure. In view of the results expressed above in Table 6 this is not considered likely.

(ii) Progestogen is in low concentration in the blood sample extracted. This is considered likely and is discussed more fully on page 53.

(iii) A substance antagonistic to the proliferative response of progestogens is present in the extract (see page 21).

Extractions of Various Blood Samples Expected to Contain Progesterone

In preparing this series of extracts, a revised procedure was attempted in the hope of simplification of the method of Allen. Also it was hoped to avoid the troublesome emulsions that were encountered at various stages of previous extractions that were performed.

Procedure

1. The plasma from a centrifuged blood sample was measured and added dropwise to 100 cc. of a mixture of 3 parts alcohol to 1 part ether. The whole was then heated to boiling, cooled and filtered.
2. The solvent was then distilled off under reduced pressure from the filtrate from stage 1.
3. The residue was shaken with benzene in the flask where it remained after stage 2.
4. This benzene solution was washed with N/20 NaOH to removed fatty acids, and then with water.
5. The solvent was distilled off and the residue weighed. It was then dissolved in ether, cholesterol was added and implants made as in the previous extraction procedure (page 36).

Table 10

PROGESTATIONAL RESPONSE OBTAINED FROM EXTRACTS PREPARED USING A REVISED EXTRACTION PROCEDURE

Rabbit No.	Material Extracted and Implanted	Uterine Response	Equivalent Blood Volume
C61	Plasma preg. cows (no. 1)	0	2 cc.
	Residue after above plasma was extracted	0	-
C62	Blood cells (preg. cow's)	0	5 cc.
	Same blood cells extract with additional cholesterol	0	4 cc.
C54	Plasma (preg. cow)	0	4 cc.
	Same plasma extract with additional cholesterol	0	3 cc.
142	Plasma (pseudopregnant rabbit)	1	35 cc.
112	Plasma (pseudopregnant rabbit)	0-½	1 cc.
		1	3 cc.
		1	25 cc.
211	Plasma (pseudopregnant rabbit, extracted to stage 1 only)	0-½	1 cc.
		0	5 cc.
		0	55 cc.

Table 10 lists the experiments that were performed using this modified extraction procedure. In extract C61, stage 1. only of the revised extraction procedure was carried out. Cholesterol was added and the solvent distilled off, leaving an oily residue from which implants were made. The second implant in this animal (C61) consisted of 5 to 10 milligrams of the residue that remained after the plasma was extracted and filtered.

It was found in all the experiments listed in Table 10 that this revised procedure gave residues which were very oily. This made it very difficult to make implants which bore a quantitative relationship to the tissue extracted. Moreover it was not possible to insert these implants into the lumen of the uterus without loss.

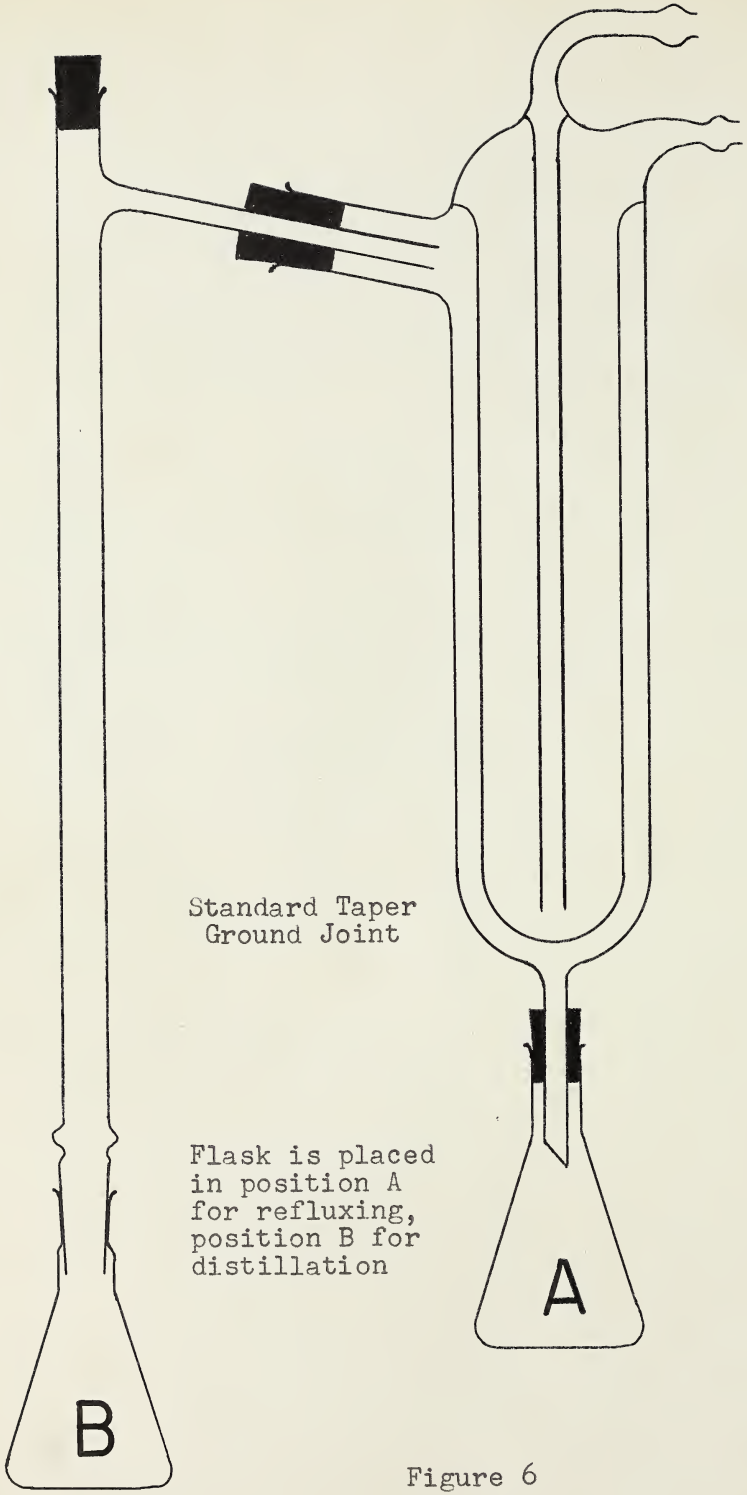

Standard Taper
Ground Joint
Flask is placed
in position A
for refluxing,
position B for
distillation
A
B

Figure 6

B. Chromatographic Procedure for the Extraction of Progestogens from Blood

A paper dealing with the extraction of testosterone and related steroids from various tissues was published by L. T. Samuels in 1947 (39). These steroid substances, one of which was progesterone, had been incubated with the tissue, (the nature of which was not stated in the paper). The procedure which he used appeared to result in a comparatively pure extract. Moreover, it appeared simpler than methods reported in the preceding section of this thesis.

Procedure

A measured amount of plasma from 10 cc. of centrifuged blood is added to approximately 20 cc. of distilled water in a 50 ml. erlenmeyer flask. The resulting diluted plasma is boiled under reflux for 20 minutes to one-half an hour using the apparatus illustrated in Fig. 6, and the flask is then cooled. Approximately 15 cc. ether is added and the flask containing boiled plasma, precipitated protein and ether is stoppered. Flask and contents are then shaken and the ether layer is allowed to settle out. Should an emulsion form, as is frequently the case, it is readily broken by placing the stoppered flask in a 250 ml. brass centrifuge cup and spinning the whole at 2,000 r.p.m. The centrifuge cups used had a rubber pad in the bottom, and as a further safeguard against breakage, about 50 cc. of water were added to each cup.

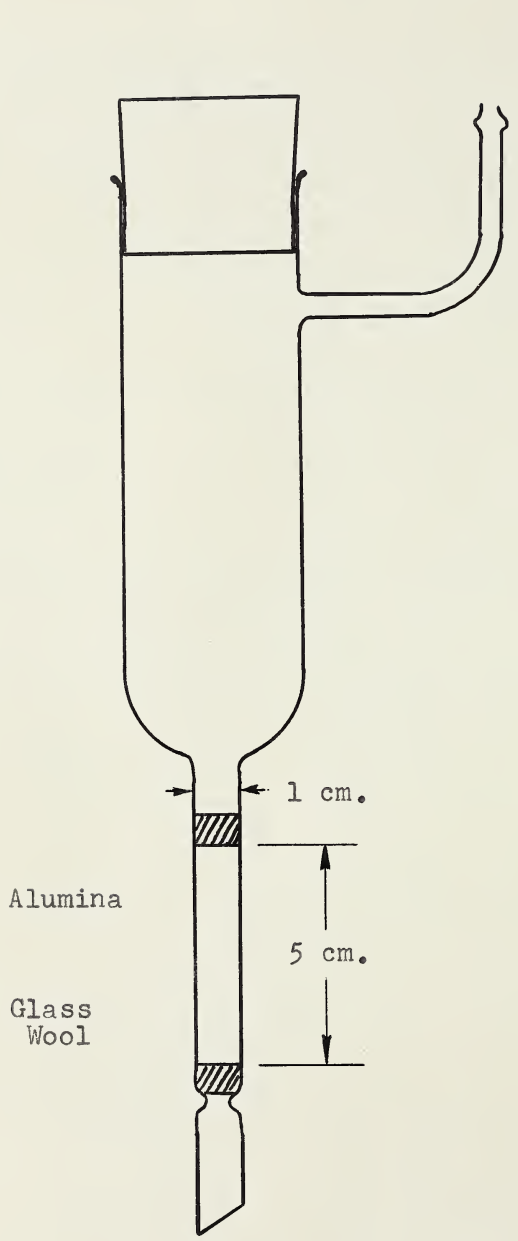


Figure 7

The ether layer was removed into a glass-stoppered flask by suction and the whole ether extraction procedure was repeated four times. It was usually found that centrifugation, if necessary at all, was required only on the first extraction.

The combined ethers from the previous extractions were concentrated by distillation through the condenser that had previously been used when heating under reflux (Fig. 6). Any residue then remaining in the flask was dried by heating on a boiling water bath under the vacuum from a water aspirator. Petroleum ether (20 cc.) was then added and allowed to stand in the flask for a minimum of four hours ar room temperature. In order to ensure that the contents were dissolved in the petroleum ether, the flask was carefully shaken several times during this interval.

A column was prepared as illustrated in Fig. 7 and packed to a depth of 5 cm. with Alumina (Alcoa Activated Alumina). A plug of glass wool was placed at either end of the column as shown in the diagram.

Through this, the following solutions were passed in the order indicated:-

(1) 10 cc. petroleum ether

(2) The 20 cc. portion of petroleum ether containing the substance under investigation

(3) 30 cc. petroleum ether

(4) 15 cc. of a 10% solution of chloroform in petroleum ether

(5) 75 cc. of a 25% solution of chloroform in petroleum ether

(6) 50 cc. of chloroform

Samuels stated that the hormone was usually found in fraction 5. This was confirmed in an experiment discussed in the section of this thesis dealing with colorimetric work.

Fraction 5 was then distilled to dryness using the apparatus previously described. The residue was then dissolved in ether or alcohol, cholesterol added and the whole washed with further solvent into weighed 10 ml. beakers. The solvent was then evaporated, using a heat lamp from above the beaker. This procedure effectively served to prevent creeping of the ether over the lip of the beaker.

A waxy residue remained and this was scraped together using a micro spatula. This was made from a steel knitting needle, ground flat and retempered. It was found that two implants totalling 1.7 to 1.8 mg. in weight could be scraped from a residue weighing 2.5 to 2 mg.

The procedure described above was used by the author in more than 30 extractions. It was noted during the first few of these that the ether layer could not be removed completely without at the same time sucking over some of the substratum. Therefore, in order to reduce the area of contact between the two layers and thus obtain a more complete separation of the ether-contained hormone, an attempt was made to carry out the precipitation and ether extraction stages in glass-stoppered test tubes. Five cc. portions of plasma were

Table 11

PROGESTATIONAL RESPONSE OBTAINED USING A CHROMATOGRAPHIC EXTRACTION PROCEDURE

Rabbit No.	Material Implanted	Uterine Response
1150	Extract of Rabbit Plasma with Added Prog. (3.5 ug./cc.)	
	(1) Equivalent to 77 ug. Prog.	4
	(2) Equivalent to 21 ug. Prog.	4
1150	Pseudopregnant Rabbit Blood	
	(1) Equiv. to 26 cc. Plasma	1
	(2) 4 ug. 1% Prog. in Chol.	0 - 1
1142	(1) Equiv. to 20 cc. Plasma	½
	(2) 2.6 ug. 1% Prog. in Chol.	½ - 1
276	(1) Equiv. to 2.5 cc. Plasma	1
	(2) 9 ug. 1% Prog. in Chol.	1 - 2
282	Human Pregnancy Blood	
	(1) Equiv. to 2.5 cc. Plasma	½ - 1
	(2) Equiv. to 0.8 cc. Plasma	0
	(3) 5 ug. 1% Prog. in Chol.	1

placed in test tubes and heated in a boiling water bath for periods up to one hour. The tubes were then cooled, ether was added and the contents shaken.

This procedure resulted in the formation of a stable emulsion in every case. In one sample that was centrifuged at 2,500 r.p.m. for an hour, the resulting clear ether layer amounted to only a small fraction of the total volume originally added.

Two samples of plasma were similarly diluted and the proteins precipitated with sodium tungstate. Again, ether extraction gave a stable emulsion.

In all subsequent extractions therefore, the procedure outlined above, in which plasma was boiled under reflux after dilution, was used.

Table 11 lists the results obtained in several experiments in which samples of plasma were extracted and the extracts submitted to assay in the rabbit uterus. The results of these few experiments are similar to those obtained using other extraction procedures and quoted above.

It should be noted that the extracts implanted into rabbits 1142 and 1150 were prepared from blood samples from weakly pseudopregnant rabbits. That is to say, on laparotomy prior to taking the blood sample, only a few small corpora lutea were found to be present in the ovaries of these rabbits. This fact probably accounts for the low levels of progestogen indicated by the uterine response.

EFFECT OF CONCENTRATIONS OF PROGESTERONE OTHER THAN ONE PERCENT IN IMPLANTED MIXTURES WITH CHOLESTEROL

Prior to my beginning this work, Dr. Höhn had begun experiments using 1% mixtures of progesterone and cholesterol. In several experiments which he performed using 0.2% and 5% concentrations of progesterone in cholesterol, neither mixture appeared to be more effective in producing the progestational response than was the 1% concentration previously used. Therefore the problem was continued using this concentration, realizing however, that some time later the final conclusions might have to be modified and some of the work, dealing with the dose-response curve, repeated.

When, however, an assay procedure was drawn up it became apparent that the concentration of progesterone in an unknown mixture could only by accident be 1% with respect to the cholesterol which was added to this mixture. This made it necessary to investigate the progestational effectiveness of various concentrations of progesterone in cholesterol which contained a given dose of progesterone just above the threshold dose.

Two groups of rabbits were implanted so that all of the rabbits in the group received the same dose of progesterone. Each animal in the group was given three implants,

Table 12

RESPONSES TO IMPLANTS CONTAINING FIVE MICROGRAMS OF PROGESTERONE AND VARYING CONCENTRATIONS OF CHOLESTEROL

Rabbit	Progestational Response		
	5% Prog.	1% Prog.	0.2% Prog.
1	2	3	3
2	1 - 1½	3	3
3	1	3	3

Table 13

RESPONSES TO IMPLANTS CONTAINING TWO MICROGRAMS OF PROGESTERONE AND VARYING CONCENTRATIONS OF CHOLESTEROL

Rabbit	Progestational Response		
	5% Prog.	1% Prog.	0.2% Prog.
4	0	0	2
5	0	0	1½

having a different concentration of progesterone in each of them. This means in effect that a relatively large implant was required to hold the test doses of progesterone in 0.2% mixture, while a smaller implant held the same doses in 5% mixture. Tables 12 and 13 list these experiments and their results.

Although only five animals were implanted, the results obtained from them were consistent. It appears that there is a difference between the effect produced by the three concentrations. The 5% mixture is less, and the 0.2% more, effective than the 1% mixture in the production of progestational proliferation.

These results were unexpected. It had been found that whenever pellets of 1% progesterone in cholesterol were implanted, they lost approximately 15% of their weight during the four days that they remained in the uterus. It was then assumed that this loss of weight represented solution of the outer layer of the implant in the uterine fluids. To support this conclusion was the fact that the implants recovered from the uterus after remaining four days in situ still retained their original shape, although sharp edges present at implantation could be observed to have become somewhat rounded. This would mean that in segments containing the more concentrated mixtures, the endometrium would be exposed to a higher concentration of progesterone during the four days that the implant was present. This in turn would be expected

to result in a greater degree of proliferation in the case of the more concentrated implants.

Since this was not found to be the case it is possible that by some means all, or most of the progesterone in the more concentrated implants was dissolved out early in the period that the implant remained in the uterus. This would result in the endometrium being subjected to an adequate concentration of progesterone for a time, but the exposure to progesterone was of too short duration to cause a proliferation. With larger implants of a weaker concentration, release of progesterone from the cholesterol would be slower and more prolonged, allowing proliferation to occur. In support of this concept is the fact, as previously noted, that implants of pure progesterone were ineffectual, presumably due to its rapid destruction.

Experiments designed to clear up this point are planned. Implants which have produced a given effect in one animal will be transplanted into the uterus of a second rabbit and if necessary a third, to determine how much progestational activity remains in implants of various sizes. The degree of activity in each rabbit will be compared in each case, and in the same uterus with fresh unused samples of the same mixture. Also implants will be left in situ for various periods up to ten days or so, and observation made of the time at which the progestational response begins to subside. If the explanation given above is correct, large

implants of weak progesterone concentration should show a prolonged effect, and small implants of stronger concentration a response of shorter duration.

The results in table 13 are so striking that additional experiments should be performed in order to determine the concentration of progesterone in mixtures with cholesterol which will give the smallest threshold dose. From the two experiments in this table it would appear that this optimum progesterone concentration is significantly below the 1% level.

TRIAL BLIND ASSAYS OF PLASMA SAMPLES WITH ADDED PROGESTERONE

As a test of the procedure proposed above for the assay of progestogen, the following experiments were carried out.

Eleven 50 ml. flasks were lettered and into nine of them were placed accurately measured aliquots of a solution of progesterone in alcohol. This was done by the author, selecting the lettered flask for each aliquot by lot. A record was kept as to which flask contained the various progesterone doses used. Dr. Höhn then placed five cc. plasma samples in four of these flasks which he selected by lot and replaced the letters by numbers 1,2,3,4. His record of the correlation between letters and numbers was then sealed and was not opened until the tests were completed. Thus neither of us knew the progesterone content of the plasma samples except that the values lay between 0 and 40 micrograms.

These four samples were then extracted using the chromatographic procedure outlined above and to each of the four extracts so obtained, two milligrams of cholesterol was added. Implants were made from the residue and assayed in the rabbit uterus along with implants containing known amounts of progesterone with results as indicated below.

Assay I

Implants		Response		Implant Residue
Unknown Extract	Known (1% P in C)	Unimplanted Control	Implanted Segment	
0.3 mg. 1.2 mg.	 2 ug. Prog. 10 ug. Prog.	0	$\frac{1}{2}$ 1 - 1$\frac{1}{2}$ 2 3	All Residues Recovered

Estimated Prog. in implant = 1 to 2 ug. in 1.2 mg.

Total weight of extract = 2 + mg. (in 5 cc. plasma)

Therefore Prog. content of sample = 2 to 4 ug.

Actual value = 4 ug.

Assay II

Implants		Response		Implant Residue
Unknown Extract	Known (1% P in C)	Unimplanted Control	Implanted Segment	
0.3 mg. 1.05 mg.	 2 ug. Prog. 10 ug. Prog.	0	$\frac{1}{2}$ 1$\frac{1}{2}$ 1$\frac{1}{2}$ 4	All Residues Recovered

Estimated Prog. in implant = 2 ug. in 1 mg.

Total weight of extract = 2.5 mg. (5 cc. plasma)

Therefore Prog. content of sample = 5 ug.

Actual value = 5 ug.

Assay III

Implants		Response		Implant Residue
Unknown Extract	Known (1% P in C)	Unimplanted Control	Implanted Segment	
0.2 mg.		0	0	Not Rec.
0.6 mg.			½ - 1	Recovered
	2 ug. Prog.		0	Not Rec.[1]
	10 ug. Prog.		4	Recovered

Estimated Prog. in implant = 2 ug. in 0.6 mg.

Total weight of extract = 2.6 mg. (5 cc. of plasma)

Therefore Prog. content of sample = 8.6 ug.

Actual value = 6 ug.

Assay IV

Implants		Response		Implant Residue
Unknown Extract	Known (1% P in C)	Unimplanted Control	Implanted Segment	
0.25 mg.		0	0	
1.1 mg.			1	All Residues
	2 ug. Prog.		1 - 1½	Recovered
	10 ug. Prog.		3	

Estimated Prog. in implant = 1 to 2 ug. in 1.1 mg.

Total weight of extract = 2.4 mg. (5 cc. of plasma)

Therefore Prog. content of sample = 2.4 to 4.8 ug.

Actual value = 25 ug.

[1]Since a residue was not recovered this implant was discarded. The progesterone content of the sample was estimated from the responses of the two implants where residues were recovered.

In the first three examples the recovery indicated is quite good. In assay IV, the difference between estimated progestogen content and actual progestogen content is so great as to be possibly due to some gross error in adding progesterone to the sample or in the extraction procedure.

In considering the blood levels of progesterone that might be expected in unknown samples, the following factors indicate that these levels might usually be low:

(1) Haskins (20), using the McGinty procedure, was not able to obtain a strong response from extracts of the blood of pregnant women.

(2) Hooker and Forbes have placed the maximum solubility of progesterone in plasma at 8 micrograms per cc. (23)

(3) Riegel (36) found that when radioprogesterone was administered to rats it was rapidly metabolized and removed from the blood.

(4) In experiments tabulated on pp. 39 and 45, also in one experiment not reported, in which an extract of pregnancy plasma was implanted in oil contained in a gelatine capsule, the degree of proliferation observed was usually slight.

In the first three examples the recovery indicated is quite good. In assay IV, the difference between estimated progestogen content and actual progestogen content is so great as to be possibly due to some gross error in adding progesterone to the sample or in the extraction procedure.

Table 14

UTERINE RESPONSE TO FILTER PAPER IMPLANTS

CONTAINING PROGESTERONE

Rabbit No.	Implant	Uterine Response
C 56	4 segments implanted, each with ½ sq. cm. filter paper containing 23 ug. Prog.	Identical with Control
C 59	Filter paper + 23 ug. Prog. " " + 11.5 ug. Prog. " " + 3.5 ug. Prog. " " + 2.3 ug. Prog.	½ ½ ½ 0
1142	20 ug. Prog. (1% in Chol.)	½ - 1
1145	33 ug. Prog. (1% in Chol.) 22 ug. Prog. " 11 ug. Prog. "	½ - 1 ½ - 1 1

EXPERIMENTS WITH OTHER TYPES OF IMPLANTS

During earlier extraction experiments, the progestogen containing residues were usually obtained in the form of a viscous oil. This was very difficult to handle and on implanting these residues, losses occurred. Freezing them did not satisfactorily solve this problem since they would quickly thaw as they were being inserted into the uterine lumen.

Therefore, attempts were made to find some other means of implanting progestogens which would not result in a significant decrease in sensitivity of a bioassay based thereon.

A. Filter Paper Implants

Solutions of progesterone, and of progesterone and cholesterol in a 1:99 ratio were evaporated on rectangular pieces of filter paper of varying size. The solvent used was ether or alcohol. These paper rectangles were then cut into small portions containing a suitable weight of progesterone, and were rolled up and implanted into the uteri of primed rabbits. An unimplanted portion of the uterus of each implanted rabbit served as a control. Table 14 shows the proliferative response obtained from the implanted rabbits. It appeared that implants prepared in this way were not more effective, and probably less so in

Table 15

UTERINE RESPONSES TO ALUMINA COLUMNS

CONTAINING PROGESTERONE

Rabbit No.	Implant	Uterine Response
932	100 ug. Prog. 5 ug. Prog.	0 0
949	10 ug. Prog. 5 ug. Prog.	0 0
922	upper half of column[1]) 2 mg. lower " " ") Prog.	1 0

[1] A solution of approximately two mg. progesterone in benzene was poured through the column which was then divided and implanted into Rabbit 922.

producing progestational proliferation than solid implants of 1% progesterone in cholesterol.

B. Implanted Micro-chromatographic Columns Containing Progesterone

Small glass tubes, about 60 mm. in length and having a bore of two to three mm. were packed with alumina (Alcoa Activated Alumina) to a depth of one cm. These tubes had an enlarged upper end which served as a funnel when adding liquids. Solutions containing progesterone were poured through, and the entire contents of the tube were then extruded into the lumen of the uterus of a primed rabbit. These implants were then treated exactly as were the implants of one percent progesterone in cholesterol discussed in the previous sections of this thesis. Table 15 shows that the only positive response obtained from these implants was that due to a very large dose of progesterone.

Since neither of these procedures appeared to offer greater sensitivity than that using 1% progesterone in cholesterol, and because of the technical difficulties encountered in quantitatively transferring the progesterone solution to the filter paper without loss work was discontinued on this type of implant. It is possible, however that a suitable absorbent might be found which in combination with a suitable technique would enable implants of this type to be used.

Table 16.

IMPLANTS OF PROGESTERONE (1% IN CHOLESTEROL)

CONTAINED IN GELATIN CAPSULES

Rabbit No.	Implant		Uterine Response
951	11.0 ug. Prog. 5.0 ug. Prog. 5.5 ug. Prog.	(in capsule) (in capsule) (1% in chol.)	½ - 1 ½ ½
1295	14.0 ug. Prog. 4.4 ug. Prog. 10.0 ug. Prog. 4.4 ug. Prog.	(in capsule) (in capsule) (1% in chol.) (1% in chol.)	½ - 1 0 1 0
278	20.0 ug. Prog. 8.0 ug. Prog. 4.0 ug. Prog. 2.0 ug. Prog.	(in capsule) (in capsule) (in capsule) (in capsule)	1 1 ½ - 1 ½
277	20.0 ug. Prog. 8.0 ug. Prog. 4.0 ug. Prog. 2.0 ug. Prog.	(in capsule) (in capsule) (in capsule) (in capsule)	1 - 2 ½ ½ - 1 ½

C. Implants in Gelatin Capsules

It is possible using micropipettes of the type described by Paul L. Kirk (24) to measure accurately quantities of liquid between 1λ and 100λ in volume.[1] In preliminary trials using a 70λ pipette, solutions of progesterone and cholesterol (in a ratio of 1:99 by weight) in alcohol were placed in open gelatine capsules having a volume of 100λ and evaporated to dryness. The evaporation was carried out by placing the filled capsule in a vacuum dessicator at 25 to 30°C for one half to one hour, and it was found that the solvent was completely removed by this procedure without apparently creeping over the top of the capsule.

Capsules were prepared containing appropriate amounts of progesterone and cholesterol and were implanted into primed rabbits. One end of the capsule was left open. Table 16 shows the endometrial proliferation induced by them. The implants in animals 951 and 1295 were made from the same mixture of 1% progesterone in cholesterol. Those in the capsules were weighed out in the same manner and at the same time as the control implants. They were then placed in the respective capsules and dissolved in a drop of ethanol which was removed by evaporation. In the last two animals the implanted capsules were prepared by evaporating in them aliquots of a solution of progesterone and cholesterol in ethanol. This solution was made from

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[1] The microliter (λ) is used as the unit of volume for measurement of small amounts of liquids. (1000 λ = 1 ml.)

pure crystalline progesterone and pure cholesterol, dissolved in a 1:99 ratio as in previous experiments.

The responses observed cannot be considered as completely satisfactory, since the relatively high doses of progesterone produced no higher response than two. However it appears that the threshold is but little changed if at all, in comparison with that obtained with 1% in cholesterol implants.

Since this method offers a means whereby implants may be easily prepared and handled, and in view of the results from the four experiments quoted above, it is considered that further studies of this type of implant are warranted.

Two animals were also implanted with capsules containing progesterone dissolved in peanut oil. McGinty had shown (29) that when dissolved in peanut oil, progesterone injected into the primed rabbit uterus was capable of producing endometrial proliferation in very low doses. The results obtained were inconclusive and do not warrant comment. Further experiments of this nature are planned.

EXPERIMENTS DEALING WITH THE ANTAGONISM BETWEEN OESTROGENS AND PROGESTOGENS

It has been pointed out above (page 22) that the possible presence of antagonists must be considered in any assay for progestogens using the endometrial response of the rabbit as its basis. In the introduction to this thesis, it has been shown that oestrogens are antagonistic to progesterone insofar as its progestational activity is concerned. Moreover, it was found that the dose of oestrogen required to inhibit completely the progestational response when both of these substances were given together, was in the neighborhood of 1/75 the weight of the progesterone injected.

The work reported below was carried out in order to evaluate better the effect of oestrogens which might be present in implants containing progestogen and applied locally to the rabbit uterus.

The antagonism between oestrone and progesterone was accordingly investigated under the following conditions of administration of the two hormones:

(1) Oestrone implanted into the uterus and progesterone injected subcutaneously.

(2) Oestrone and progesterone both implanted into the uterus.

(3) Oestrone injected subcutaneously and progesterone implanted into the uterus.

As will be shown below, inhibition of the progestational response could be produced in all three sets of conditions. This pointed to the endometrium as the site of the antagonism between oestrone and progesterone.

The quantitative aspects of this inhibition were studied by performing a series of experiments for each set of conditions, to determine the progesterone/oestrone ratio for inhibition, i.e., the minimal quantity of oestrone required to inhibit the effect of a given quantity of progesterone.

As a general rule, the minimal dose of a hormone that is required to produce an effect is less when the hormone is applied directly to the tissue involved in that effect, than when the same hormone is administered systemically. This is true for the progestational response of the primed rabbit endometrium. It has been shown (page 27) that approximately 2 micrograms of progesterone (given as a 1% mixture in cholesterol) will produce a proliferative response in 50% of the animals receiving that dose. The corresponding systemic dose may be taken as about 500 micrograms, giving a local/systemic dose ratio of 1/250. Emmens (15) reports a similar situation with respect to the action of oestrone in the vaginal response in the mouse. Median effective doses for local and systemic administration were 0.00029 micrograms and 0.075 micrograms, a local/systemic ratio of 1/260.

These facts led to the expectation that in the present antagonism experiments the response to a given dose of progesterone could be inhibited by a much smaller dose of oestrone, when that oestrone was applied to the uterus, than when injected subcutaneously. It soon became clear, however, that this was not the case and that similar amounts of oestrone were required under both conditions of administration, i.e., the difference between the local and systemic effective doses for antagonism, in this case, was relatively slight. This raised the possibility that in the rabbit uterus the local and systemic effective doses for oestrogenic effect of oestrone were not very different. Experiments to determine these doses were therefore performed. They showed that under the conditions used, the local and systemic doses were, in fact, close together. Since this result was somewhat unexpected, it was felt desirable to determine the local and systemic dose on the same preparation of another true oestrogen. Ethinyl oestradiol was used for this purpose.

A. <u>The Effective Dose of Systemic Oestrone on the Rabbit's Endometrium</u>

Mature, non-pregnant rabbits were ovariectomized and left for four weeks to allow considerable atrophy of the uterus to occur. A segment of uterus to serve as a control was then removed and fixed. Oestrone in olive oil, 1 microgram per cc. was then injected subcutaneously twice daily for

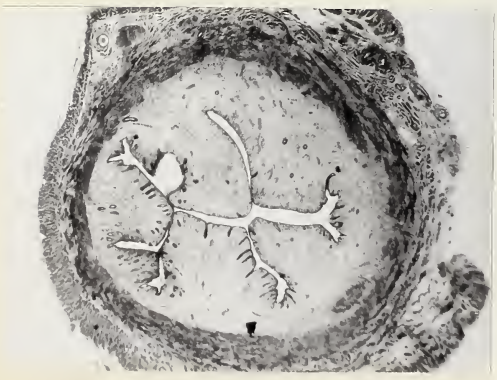
Table 17.

EFFECTIVE SYSTEMIC DOSE OF OESTRONE ON ENDOMETRIUM OF OVARIECTOMIZED RABBITS.

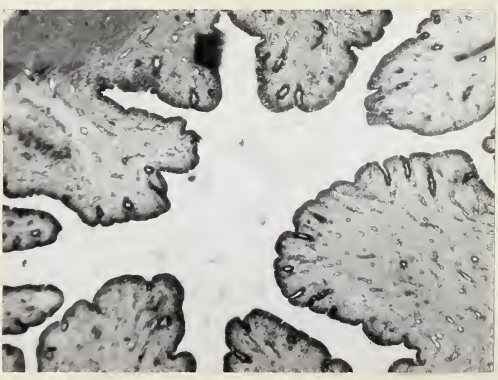
Rabbit No.	Total Dose of Oestrone (ug.)	Oestrogenic Effect
143	10.0	+ +
28	10.0	+ +
144	4.8	+ +
147	4.8	+ +
177	4.0	+ +
187	4.0	-
147a	3.0	+ +
203	3.0	+ +
158	3.0	+ +
175	3.0	+ +
170	2.4	+
205	2.0	+ +
206	2.0	+
216	1.0	-
A28	1.0	-

FIGURE 8.

Control (x 30)



Oestrone (x 50)



four days, in the amounts required to produce the total doses indicated in Table 17. The animal was then killed and a segment of the uterus was fixed and sections from it were made.

The changes of the histological appearance in the rabbit endometrium produced by oestrogen do not appear to have been studied in any detail previously. Three endometrial effects were found on comparison with the control segments showing the atrophic endometrium.

(1) Increase in size and number of blood vessels

(2) A considerable increase in the thickness of the endometrium associated with a greater separation of the stromal nuclei from one another. Both these appearances are presumably due to oedema of the endometrial stroma

(3) An increase in glands in the most superficial layer as well as the appearance of glands in the deeper layers of endometrium where they are entirely absent in the atrophic endometrium (Figure 8).

In the various tables below, oestrogenic effects are indicated as - + for a minimal and + or + + for more marked effects. As indicated in Table 17, the minimal effective dose is about 2 micrograms.

The difference between the effects from doses of 2 and 1 micrograms was quite clear-cut. It is of interest that the minimal dose for endometrial effects in mature rabbits is in agreement with a minimal dose of about 1 microgram of

oestrone which was reported to produce definite increase of uterine weight in immature rabbits by M. K. McPhail (31).

B. The Effective Dose of Intra Uterine Oestrone on the Rabbit's Endometrium

Implants of 1% oestrone in cholesterol were prepared by dissolving oestrone and cholesterol in ether, evaporating the solvent and fusing the residue. The oestrone content of these mixtures was checked by testing their effect on vaginal smears of ovariectomized mice, after subcutaneous injection.

Rabbits ovariectomized four weeks previously received intra uterine implants of these mixtures in the manner already described (page 26). A control segment (C 1) was removed at the time of implantation. Four days later the animal was killed and a second control segment (C 2) as well as the implanted segments were removed. Sections from these various segments were prepared.

The evaluation of responses to intra uterine implants was more difficult than that of responses following systemic oestrogen administration. Endometrial thickening and separation of stromal nuclei was found to some degree in ligated uterine segments from rabbits which had not received any oestrogen and can, therefore, be produced by mechanical interference alone. It was also found difficult to produce strictly localized oestrogenic effects. If the total weight of implanted oestrone was well above 1 microgram, gland development was often increased in the C 2 control segment nearly

Table 18.

EFFECTIVE INTRA UTERINE DOSE OF OESTRONE

(1% IN CHOLESTEROL IMPLANTS)

ON ENDOMETRIUM OF OVARIECTOMIZED RABBITS

Rabbit No.	Dose of Oestrogen (ug.)	Oestrogenic Effects			Gain or Loss of Implant Weight
		Implant	C 1	C 2	
127	3	+ +			- 33%
	2	+ +			- 50%
	1	+ +	-	+	- 60%
134	3	+ +			---
	2	+ +			0
	1	+ +	-	+	0
1134	1	+			---
	½	+	-	- +	+ 140%
1147	1	+			+ 40%
	½	+	-	-	---
145	1	+	-	-	---
146	½	- +	-	-	---
208	½	- +	-	-	---
212	½	- +	-	-	0
150	½	-	-	-	- 20%
162	½	-	-	-	---
163	½	-	-	-	---

as much as in the implanted segments. This was attributed to the effect of circulating oestrone absorbed from the implants. However, it was found that doses of oestrone of 1 and 2 micrograms produced a distinct thickening of the layer of epithelial cells lining the inside of the endometrium. In spite of these difficulties of interpretation, it can be concluded from the results shown in Table 18 that the minimal local dose is ½ to 1 micrograms, giving a local to systemic dose ratio of only ¼ to ½. This finding raised the possibility that extremely little oestrone was, in fact, absorbed from the implants and experiments were performed to investigate this possibility.

Two groups of fifteen implants, each weighing 0.2 mg. were prepared from a 1% mixture of oestrone and cholesterol. The total weights of the implants in these groups were 3.0 and 3.1 mg. respectively. Thus, each implant contained two micrograms of oestrone. The fifteen implants in one of these groups were placed in separate uterine segments (using a total of three rabbits) and left for four days. At the end of this period, thirteen of the implants were recovered. This group and the original group were then forwarded by Dr. Höhn to Dr. Robson for the assay of oestrogen content. The results of this assay are not as yet available.

The final column in Table 18 gives the percentage gain or loss of weight that was found when the weights of the recovered implants were compared with the original weight before implantation. Blanks in this column signify that the

Table 19.

EFFECT OF SYSTEMIC ETHINYL OESTRADIOL ON ENDOMETRIUM OF OVARIECTOMIZED RABBITS

Rabbit No.	Total Dose of Oestrogen (ug.)	Oestrogenic Effect
249	2	+ +
250	2	+ +
225	1	+
224	1	- +

Table 20.

EFFECT OF INTRA UTERINE DOSES OF ETHINYL OESTRADIOL (1% IN CHOLESTEROL IMPLANTS) ON ENDOMETRIUM OF OVARIECTOMIZED RABBITS

Rabbit No.	Dose of Oestrogen (ug.)	Oestrogenic Effects			Gain or Loss of Implant Weight
		Implant	C 1	C 2	
230	3	+ +			- 67%
	2	+ +			- 70%
	1	+ +	-	+ +	---
233	3	+ +			---
	2	+ +			---
	1	+ +	-	+ +	- 60%
1107	1	- +	-	-	0
1148	1	- +	-	-	- 50%
	½	- - +			---
1137	½	- +	-	- +	---

implant was not recovered when the uterine sections were removed. Because of the large variations, no significant conclusions can be drawn from these weight changes. However, the fact that a particular implant was recovered establishes its presence in the uterine segment and renders any responses obtained from that implant more significant.

C. The Effective Dose of Systemic and Intra Uterine Ethinyl Oestradiol

Because the systemic and local effective doses for oestrone were found to be so similar, another oestrogen, Ethinyl oestradiol was also investigated. Experiments exactly as those described above for oestrone were carried out with ethinyl oestradiol. The results are given in Tables 19 and 20. It will be seen that the minimal systemic dose is about one microgram. With intra uterine implants, oestrogenic responses are sometimes observed in the non implanted segments as was noted with oestrone. The minimal local dose is of the order of ½ microgram, giving a local/systemic dose ratio of 1:2. This is similar to the findings with oestrone and suggests that a low local/systemic dose ratio for effects on the rabbit's endometrium apply to oestrogens in general.

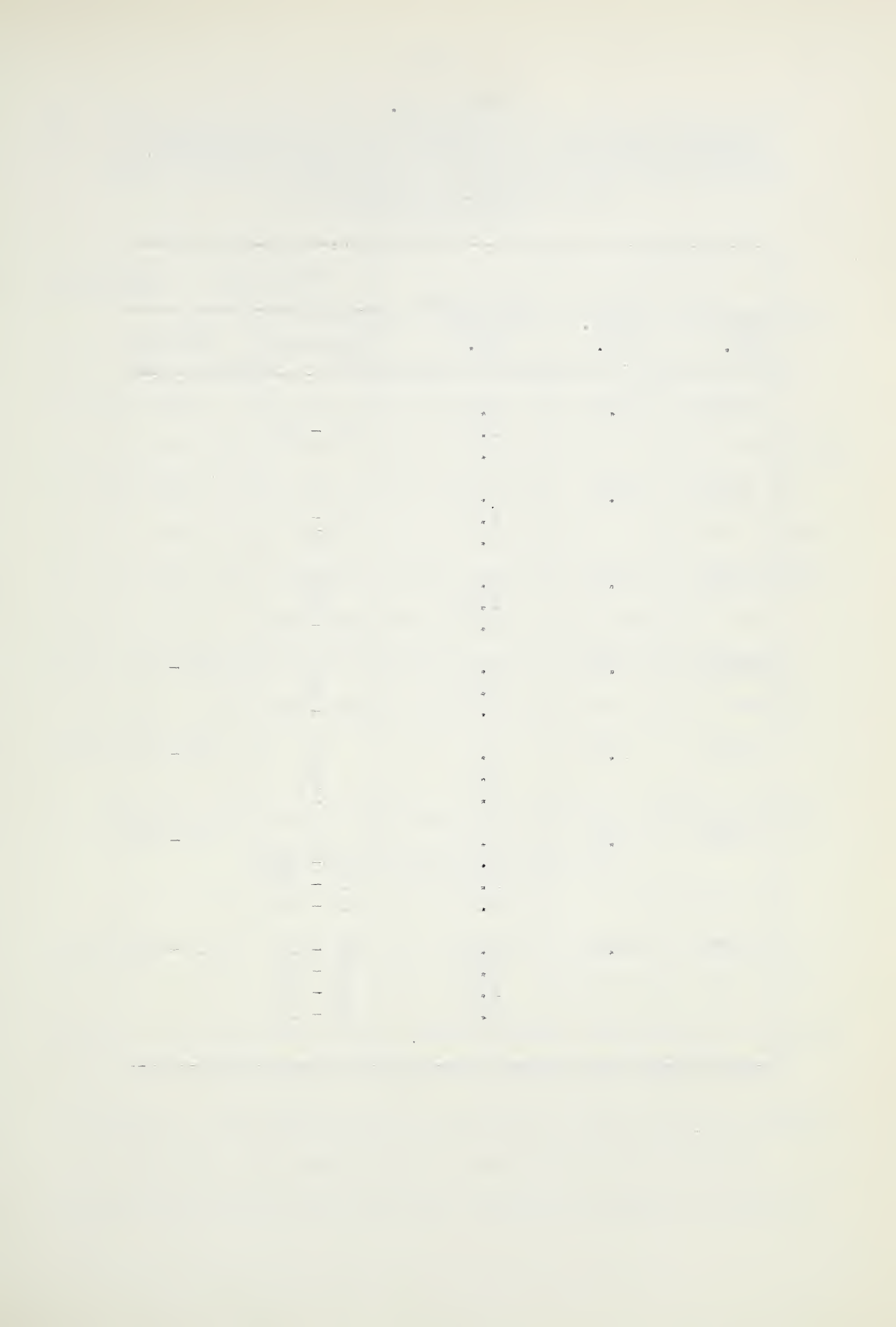


Table 21.

UTERINE IMPLANTS OF OESTRONE (1% IN CHOLESTEROL)

AND SYSTEMIC PROGESTERONE

Animal No.	Dose Prog. (mg.)	Oestrone Dosages (ug.)	Responses	
			Implanted	Control
D42	1.0	2.0 1.0 0.5	0 0 - ½ 2	3
D43	1.0	2.0 1.0 0.5	0 0 - 1 1	3
161	1.0	2.0 1.0 0.5	½ ½ 1 - 2	3
1146	1.0	2.0 1.0 0.5	2 ½ 1 - 2	3 - 4
1157	1.0	2.0 1.0 0.5	½ ½ 1	2 - 3
283	1.0	3.0 2.0 1.0 0.5	1 1 - 1½ 1 - 2 1 - 2	2 - 3
299	1.0	3.0 2.0 1.0 0.5	½ - 1 ½ - 1 0 - ½ ½ - 1	1 - 2

D. Inhibition of the Progestational Reaction by Oestrone Under Varying Conditions of Oestrone and Progesterone Administration

Technique

All the experiments were conducted on the same general plan as those to determine the dose-response curve of progesterone. Spayed female rabbits were sensitized to progesterone by daily subcutaneous injections of 10 micrograms of oestrone in olive oil for seven days. Intra uterine implants containing progesterone or oestrone or both were made and left in situ for four days. Sections from the implanted segments were examined microscopically to determine the degree of progestational proliferation of the endometrium, which was rated on the semi quantitative scale described by McPhail (page 4).

1. Uterine Implants of Oestrone and Systemic Administration of Progesterone (See Table 21)

In these experiments implants of 1% oestrone in cholesterol were inserted into separate segments of uterine horn and left in situ for four days. During this time, the animal received twice daily subcutaneous injections of progesterone in olive oil to produce the total dose indicated in the table. The histological condition of the control segment then indicates the endometrial response to the injected progesterone while the oestrone implanted segments indicate

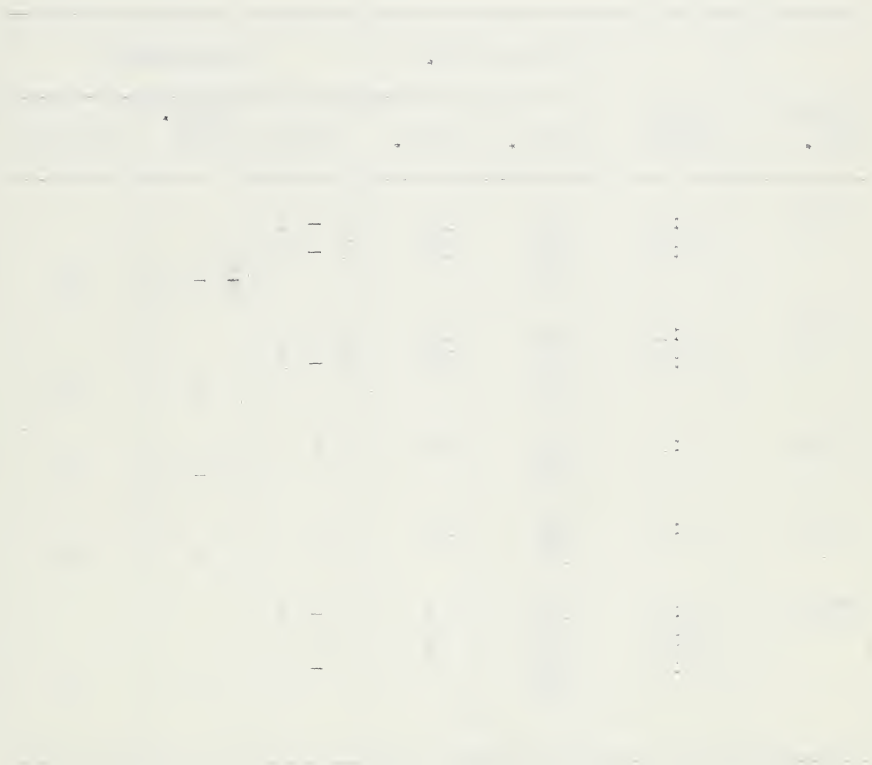


Table 22.

UTERINE IMPLANTS OF OESTRONE AND PROGESTERONE

(BOTH 1% IN CHOLESTEROL)

IN THE SAME UTERINE SEGMENT

Animal No.	P/O Ratio	Doses (ug.)		Responses		
		Prog.	Oest.	Both	Prog. Only	Control
C27	4:1	40	10	½ - 1		
	3:1	30	10	1 - 2		
		10			2 - 3	½
C38	4:1	40	10	2		
	3:1	30	10	½ - 1		
		10			2	0
C94	2:1	20	10	½		
		10			2 - 3	0
C95	2:1	20	10	0		
		10			2	0
279	4:1	12	3	1 - 2		
	3:1	12	4	1		
	2:1	10	5	0 - ½		
		10			2	0

Table 23.

UTERINE IMPLANTS CONTAINING BOTH OESTRONE AND PROGESTERONE IN CHOLESTEROL

Animal No.	P/O Ratio	Doses (ug.)		Responses		
		Prog.	Oest.	Both	Prog. Only	Control
12	4:1	20	5	2		
	3:1	20	6	1 - 1½		
	2:1	20	10	1		
		20			1 - 1½	0
20	3:1	20	6	1		
		20	10	½ - 1		
		20			2	0
291	4:1	20	5	½ - 1		
	3:1	20	6	½		
	2:1	20	10	0		
		20			1 - 2	0

to what extent this response had been inhibited by local action of the oestrone.

The results given in Table 21 indicate that the effect of 1.0 mg. of systemic progesterone is inhibited by 1.0 micrograms of oestrone acting locally. It is not considered that the recorded weight changes of the implants during the four days in uteri permit significant conclusions to be drawn.

2. Separate Uterine Implants Containing Oestrone and Progesterone (See Table 22)

In these experiments two implants, one of 1% oestrone in cholesterol and one of 1% progesterone in cholesterol, were placed in the same uterine segment. In some animals (as shown in the table), two segments were thus implanted. A further segment was given an implant of progesterone only. The results indicate that at a progesterone/oestrone ratio of 2:1, complete inhibition is obtained.

3. Intra Uterine Implants Containing Both Oestrone and Progesterone in Cholesterol (See Table 23)

Implants containing both hormones in various proportions in cholesterol were prepared in the manner previously described for the preparation of oestrone in cholesterol implants. The composition of these mixtures was such that progesterone content was always 1%, the oestrone content being 1/4, 1/3 or 1/2 of this, producing mixtures with 4:1, 3:1 and 2:1 progesterone/oestrone content. The actual dose of

Table 24.

UTERINE IMPLANTS OF PROGESTERONE (1% IN CHOLESTEROL) AND SYSTEMIC OESTRONE

Animal No.	Total Dose of Oestrone (ug.)	Doses of Progesterone (ug.)	Responses	
			Implanted	Control
165	20	10	0	0
		5	0	
		2	0	
		1	0	
153	20	10	2 - 3	0
		5	½ - 1	
		2	1 - 2	
152	20	10	2 - 3	0
		5	1	
		2	½ - 1	
156	20	10	3 - 4	0
		5	1	
		2	0	
135	20	10	1 - 2	0
		5	0	
		2	0	
		1	0	

the two hormones in each implant is shown in Table 23. It will be seen that under these circumstances also, complete inhibition of the response to progesterone requires a progesterone/oestrone ratio of 2:1, just as when separate implants of the two hormones are used.

4. Uterine Implants of Progesterone and Systemic Administration of Oestrone (See Table 24)

In these experiments, three to four uterine segments were implanted, each with a different dose of progesterone given as 1% progesterone in cholesterol, while oestrone was given by twice daily subcutaneous injection for four days to produce the total dose indicated in the table. The animals were killed, as in other experiments, after four days.

The results indicate that while 20 micrograms of injected oestrone is insufficient to inhibit in most cases the response to 10 micrograms intra uterine progesterone, it can inhibit the response to 5 micrograms progesterone. The progesterone/oestrone ratio for inhibition under these circumstances is therefore 1:4.

In the series of experiments reported above, it has been shown that the progestational response to systemic progesterone can be inhibited locally by implants of oestrone placed in the uterus. These suppress the effect of the circulating progesterone only in the uterine segment which contains the implant. This is in agreement with similar results obtained with intra uterine injections of oestradiol

benzoate by Courrier and Poumeau Delille (12). Further, these experiments also show that this local inhibition of the response to implants of progesterone occurs with adequate amounts of oestrone, present either as a separate implant or mixed with the progesterone in a common implant. It is thus clearly established that the normal progestational response of endometrial cells to progesterone which reaches them (either systemically or from the uterine lumen) can be prevented by oestrogen. Also, this inhibition occurs in the region of the cells themselves and not elsewhere in the body.

It is interesting to compare the quantitative aspects of the antagonism of oestrone and progesterone under various conditions of administration of the two hormones. From the papers of Robson (38), Gillman (16) and Courrier (11), already referred to, it appears that in the rabbit, 1/75 of a milligram or less of oestrone is sufficient to inhibit 1 milligram of progesterone when both of these substances are given systemically. A comparison may therefore be made on the following basis:

(1) The observed progesterone/oestrone ratio for inhibition when both are given systemically is 75:1.

(2) One milligram of progesterone (which is equivalent to the International Unit for progestin) given systemically will produce a degree of proliferation equivalent to that demonstrated on the eighth day of pregnancy. This is a

Table 25.

COMPARISON OF CALCULATED AND OBSERVED PROGESTERONE/OESTRONE RATIOS FOR INHIBITION OF THE PROGESTATIONAL RESPONSE UNDER VARIOUS CONDITIONS OF ADMINISTRATION

Conditions of Administration	Weights of Hormones When Complete Inhibition is Obtained			
	Calculated		Observed	
	Prog./Oest.	Ratio	Prog./Oest.	Ratio
Both systemic	1 mg./14 ug.	75:1	Basis of comparison	
Both in utero	10 ug./ 7 ug.	3:2	20 ug./10 ug.	2:1
Prog. systemic Oest. in utero	1 mg./ 7 ug.	150:1	1 mg./ 1 ug.	1000:1
Oest. systemic Prog. in utero	10 ug./14 ug.	2:3	5 ug./20 ug.	1:4

proliferation of about two to three on the McPhail scale and is given by about 10 micrograms of intra-uterine progesterone (1% in cholesterol). Therefore, it is considered that 1 mg. of progesterone is equivalent approximately to 10 micrograms of intra-uterine progesterone (1% in cholesterol).

(3) Two micrograms of systemic oestrone is equivalent to one microgram of intra-uterine oestrone (1% in cholesterol).

Table 25 shows a comparison of the calculated and observed ratios of these two hormones under the various conditions of administration. Although some differences are observed, it is considered that they are within the experimental range of error, since the experimental results upon which these ratios are calculated are comparatively few.

PROGESTATIONAL ACTIVITY OF STEROIDS OTHER THAN PROGESTERONE

In using the procedure proposed in this thesis for the assay of progestogens (page 33) to determine progesterone specifically, it is necessary in evaluating the results to consider the properties of other substances which might be present. Hohn and Robson[1] investigated the progestational activity of a number of steroids. The following were found active when implanted into the uteri of primed rabbits in the weights indicated:

Progesterone (1% in cholesterol)	1 µg.
Ethisterone (1% in cholesterol)	1 - 2 µg.
Desoxycorticosterone	2 mg.
Pregnenolone (10% in cholesterol)	100 µg.
17-Methyl dihydro testosterone	1 mg.
17-Methyl testosterone	300 µg.
Androsterone	10 mg.
Dehydroandrosterone	20 mg.

Of these Ethisterone is the only one which demonstrated activity in concentrations of the same order as progesterone. However it must be noted that of the remainder, only pregnenolone was administered with cholesterol, and this was in 10% mixture.

The following were found to be inactive:

Testosterone	40 mg.
Androstenediol	20 mg.
Androstenedione	10 mg.

---

[1] The response of the rabbit's endometrium to uterine implants of progesterone and other steroids. J. Physiol. 111, 174, (1950)

Testosterone, however, in implanted doses of 20 mg. resulted in proliferative responses in the uterus remote from the implanted segment. This is explained if we assume that the testosterone which is inactive as a progestogen is absorbed from the implanted segment. Elsewhere in the body it is converted to a substance which is active and is carried back in the bloodstream to the uterus.

COLOR REACTIONS OF PROGESTERONE

It appeared that it would be useful to study further the behavior on adsorption columns of progesterone, and various substances that were known to interfere in determinations of this hormone. This required some rapid means for identification of these substances, and since the initial experiments would be performed with pure substances, this test need not be specific.

W. F. Elvidge (14) had published work dealing with several colorimetric methods for progesterone determination in solution in ethyl oleate and two of these were investigated in the hope of using them for the above purpose.

I. Salicylaldehyde Color Reaction

Reagents - Salicylaldehyde dissolved in absolute alcohol (10% v/v).

- NaOH (7.5 N.).

Procedure Progesterone dissolved in 2 ml. absolute alcohol was placed in a 25 ml. volumetric flask. To this was added: one ml. Salicylaldehyde solution

ten ml. NaOH (7.5 N.)

The flasks were heated together with flasks containing the reagents and alcohol but no progesterone, at 80° for 10 minutes, then cooled in water. Absolute alcohol was added

to approximately 22 ml. and distilled water to 25 ml. The color was then read in a Fisher Electrophotometer. The results are shown in the following table.

Table 26.

TEST OF SALICYLALDEHYDE COLOR REACTION WITH PROGESTERONE

Sample No.	Amount Prog. (ug.)	Colorimeter Readings	
		Green Filter (525)	Blue Filter (425)
Control	0	0	70.1
1	5	2.1	76.0
2	10	1.8	76.0
3	20	3.3	79.0
4	50	2.9	78.0

A intense yellow color, perhaps due to impure reagents, developed in each flask. This, and the inconstancy of the above results, indicated the presence of color coupling compounds other than progesterone, probably in the salicylaldehyde, which was not available in pure form. This method was therefore abandoned in favor of method II below.

II. Benzoyl Chloride Method

Reagents - $ZnCl_2$ dissolved in Glacial Acetic Acid (40 g. in 100 ml.)

- Benzoyl Chloride

Procedure - Progesterone in 2 ml. chloroform was placed in a 10 ml. volumetric flask. One ml. of the $ZnCl_2$ reagent and one ml. Benzoyl Chloride were then added and the flask heated for three minutes on a vigorously boiling water bath. It was then cooled, diluted with Glacial Acetic Acid to ten ml. and read in the Fisher Electrophotometer using a red (650 mu.) filter.

In a preliminary experiment using this technique, the following results were obtained.

Table 27.

TEST OF BENZOYL CHLORIDE COLOR REACTION WITH PROGESTERONE

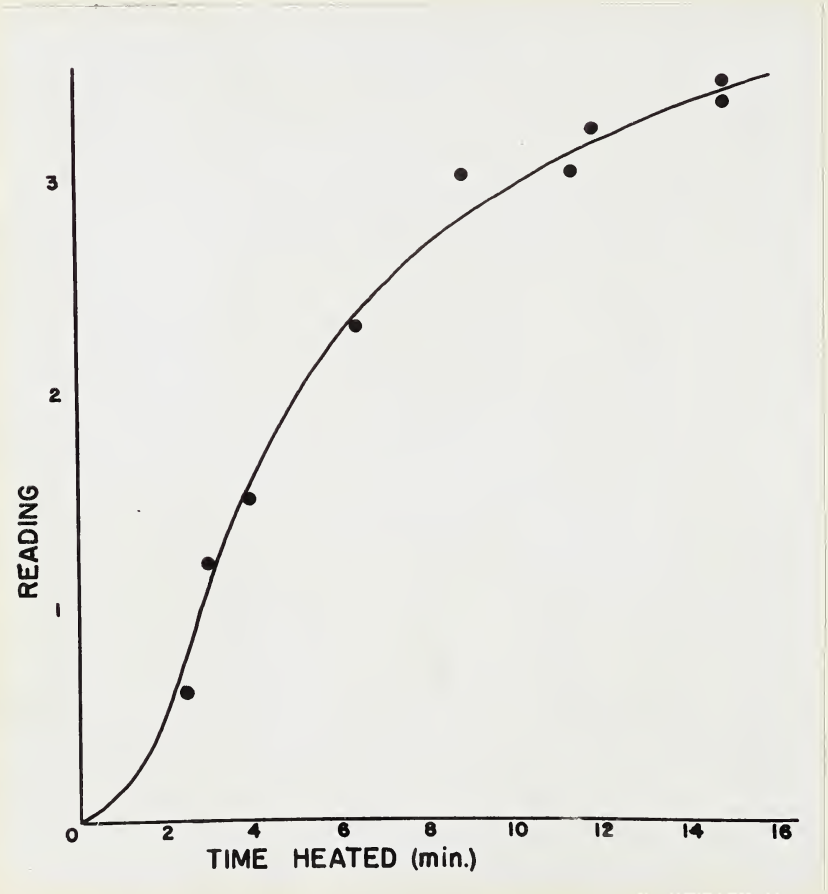
Flask No.	Prog. Solution 100 ug./ml.	Wt. Prog.	Reading (650 mu.)
22	0.4 ml.	400 ug.	37.0
61	0.2 ml.	200 ug.	20.5
68	0.1 ml.	100 ug.	10.5
75	0.05 ml.	50 ug.	5.2

These results indicated that Elvidge's belief that the method was not accurate below 500 ug. was unfounded and that further experiments were warranted.

The next three experiments gave very variable

FIGURE 9.

EFFECT OF VARYING HEATING TIMES ON THE COLOR PRODUCED BY PROGESTERONE (58 UG.) AND BENZOYL CHLORIDE.



results. However, it was discovered that during refluxing, the reagents were reacting with the rubber connector between the flask and the condenser. The condenser was replaced with one having a ground glass joint to fit the 10 ml. volumetric flasks used.

A further experiment in which two flasks were heated for twice the time (6 minutes) gave the following results.

Table 28.

EFFECT OF DOUBLING HEATING TIME ON COLOR DEVELOPED WITH PROGESTERONE AND BENZOYL CHLORIDE

Flask No.	Wt. Prog. (ug.)	Time Heated	Reading (650 mu.)
20	80	3 min.	8.3
12	80	3 min.	7.6
21	80	6 min.	10.3
84	80	6 min.	11.1

Apparently more prolonged heating gave a more intense color, although it was noted that the shade had changed somewhat also.

Therefore, a series of 58 microgram samples was heated for varying periods of time; the curve obtained is shown in Figure 9. Two blanks containing all the reagents but no progesterone were heated for fifteen minutes.

These blanks acquired a yellow color which, however, gave a reading of only 0.3 in the colorimeter.

It was concluded from this graph that a heating time in the neighborhood of ten minutes would be more suitable than the three minute time previously used.

At this time, very little Benzoyl Chloride remained and further supplies were not available. For this reason, and since it appeared more advantageous to complete experiments already in hand dealing with phases of the work discussed above, these experiments were carried no further except as noted below.

Colorimetric Test of Elution of Progesterone from an Alumina Column

A solution of 33.6 micrograms of progesterone in petroleum ether was run through a column of alumina using the procedure outlined on page 42. Fractions 4, 5 and 6 were then evaporated to dryness in 10 ml. volumetric flasks and 2 ml. chloroform was added to dissolve the progesterone. The $ZnCl_2$ reagent and Benzoyl Chloride were added as described above and the flasks were heated for ten minutes. When examined in a colorimeter, the following results were obtained in two trials: Fractions 4 and 6 - no readings higher than 0.1 were obtained

Fraction 5 - readings of 2.5 and 2.6

This indicated that most of the added progesterone had appeared in fraction 5 and little, if any, in fractions

4 and 6. This confirmed the findings of Samuels who stated (39) that in no case had progesterone been found in fractions other than 5.

SECTION III.

CONCLUSIONS

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CONCLUSIONS

It was stated above that the primary objective of this research was to obtain a procedure whereby the progestational activity of a sample of blood could be estimated. Such a procedure has been outlined, but it is considered that this procedure is subject to a number of considerations which limit the uses to which it may at this time be put.

In the section dealing with antagonism it was pointed out that oestrogens in certain concentrations will prevent, or reduce the degree of, progestational responses to implants of progestogen in the test animal. Also, oestrogens which are present in the blood sample undoubtedly play a part in the hormonal actions of progestogen on the tissues of the animal from which the sample was obtained and thus it is essential to take into account the presence or absence of oestrogens at all stages of the procedure.

However, it is noteworthy that when the progestogen is applied locally, it requires a much greater proportion of oestrogen to progestogen to inhibit the progestational response than when the progestogen is administered systemically.

Certain problems still remain to be settled, such as that of the concentration of progesterone in implants where it is mixed with cholesterol, which will produce the

greatest degree of proliferation for a given dose of progesterone.

A second important question that still remains unanswered is whether all of the progesterone in a blood sample is recovered by the extraction procedures discussed above; and if not, what proportion is lost at various stages of the procedure?

An interesting side-issue that arose and is still unsettled is why two micrograms of progesterone in a 0.2% mixture with cholesterol will exert greater progestational effect on the rabbit endometrium than will a 1% or 5% mixture.

The procedure described in this thesis for the bioassay of progestogens in blood samples will detect progesterone in such samples in absolute amounts as low as one microgram. It is specific for substances having direct progestational activity on the endometrium and it is not significantly interfered with by oestrogens in concentrations less than one-tenth of the amount of progesterone present in the sample.

SUMMARY

SUMMARY

1. Various procedures for the assay of progestogens have been reviewed. The advantages and disadvantages of each as applicable to the estimation of progestogens in blood were discussed.

2. Pellets containing progesterone mixed with cholesterol were implanted into the rabbit uterus and the progestational proliferation which they evoked was evaluated according to the semi-quantitative scale proposed by McPhail. The curve relating dosage level to response was plotted from the uterine responses to 199 of these pellets and the data was found to be significant when a statistical test was applied. A threshold dose for this response was found to be in the neighborhood of one microgram.

3. A procedure based on this response was outlined for the estimation of progestogens in blood. This was tested by the analysis of four 5 cc. samples of plasma, to which known amounts of progesterone had been added. In three of these samples, good recovery of the hormone added was demonstrated.

4. Several procedures for the extraction of progestogens from blood or plasma were tested and found satisfactory for use in the rabbit endometrium assay referred to above. Previous work on the antagonism of progesterone and oestrone was reviewed. The doses of oestrone and of ethinyl oestradiol required to produce observable oestrogenic changes in the

rabbit uterus were determined for both local and systemic administration of these two substances. The dose of oestrone required to inhibit the proliferative response to intra-uterine progesterone was also determined when administered locally and systemically.

6. The effect of concentrations of progesterone other than one percent in implanted mixtures with cholesterol was briefly examined, A 0.2% mixture with cholesterol was found to produce the progestational response in lower dosage than did the 1% or 5% mixtures.

7. Various progesterone-containing implants were tested. None was found more satisfactory than the appropriate mixtures of progesterone in cholesterol referred to above.

8. Color reactions of progesterone with benzoyl chloride and with salicylaldehyde were examined. Benzoyl chloride appeared to be a satisfactory reagent for the estimation of progesterone in amounts greater than ten micrograms. However it is not specific for progesterone.

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